The role of primary metabolism in plant resistance against herbivory: A study with the native annual *Nicotiana attenuata*

Dissertation

Zur Erlangung des akademischen Grades
Doctor rerum naturalium (Dr. rer. nat.)

Vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

von Diplom-Trophologe
Jens Schwachtje

Geb. am 14. 6. 1970 in Bietigheim-Bissingen/Württ.

Gutachter:

1. Prof. Ian T. Baldwin
2. Prof. Ralf Oelmüller
3. Prof. Dierk Scheel

Tag der Doktorprüfung: 30. 1. 2008

Tag der öffentlichen Verteidigung: 20. 2. 2008

Table of contents

1. Introduction

1.1 A plant's response to its environment is highly plastic

During their evolution, plants stepwise gained the characteristics needed to flourish optimally in different natural environments, and also to cope with the challenging sides. Genetic changes and natural selection allowed them to adapt to different ecological niches and habitats, reflected in a great variety of plant families and species. Additionally to this genotypic variety, plants exhibit highly plastic phenotypes to specifically respond to certain environmental conditions.

Abiotic environmental stresses challenge plants in various ways, e.g. UV-radiation induces production of protective color pigments, salt accumulation in soils forces plants to develop salt tolerance, and changing climates require complex systems to regulate water homeostasis and photosynthesis. Among biotic stressors are organisms, for which plants are potential food sources or habitats. They can damage their host plants to different degrees and in response plants are either forced to defend themselves or to tolerate the damage. This is achieved by various phenotypical changes that are based on altering core processes as well as secondary branches of plant metabolism.

Besides contributing to reproduction and genetic variety of plants (pollination, zoochory), insects became a threat for plants early in plant history (Labandeira et al., 1994). Developed during a long time of co-evolution, plant responses to herbivorous insects are among the most complex ongoing adaptations of plants to their environment. Because some insect populations can reach outbreak proportions and affect the yield of plants cultivated by humans for their own benefit, the study of plant-insect interactions has been of interest for researchers for more than a century.

1.2 Plant resistance against insects

In order to resist herbivore damage, plants have developed a variety of traits that are constitutively expressed or induced in response to herbivore attack. From a functional perspective, resistance comprises signalling, direct and indirect defenses, and tolerance: In response to herbivory several signalling cascades are activated that regulate the expression of resistance traits (Staswick et al., 1994; Ryan and Pearce, 2003), for example MAP kinases cascades and jasmonic acid signalling (Chini et al., 2007; Wu et al., 2007). Direct defenses

can be physical: Thorns, spikes, calcium oxalate crystals or sticky trichomes on leaves may prohibit certain insects from feeding (Franceschi and Nakata, 2005). A greater variety of direct defenses is physiological, where plants have the ability to create many different substances that affect insects: Glucosinolates, for example, are toxic secondary metabolites. These amino-acid derivatives are found in Brassicaceae and close relatives (Mewis et al., 2006); the alkaloid nicotine is produced by tobacco plants and has a toxic effect on the insect nervous system (Schmeltz, 1971); proteinase inhibitors are proteins that interact with digestive enzymes of the insect's gut and decrease the efficiency of digestion (Broadway and Duffey, 1986). When attacked by insects, plants synthesize volatile organic compounds, such as bergamotene or volicitin, that act as indirect defenses. They attract other insects for which herbivorous insects are a prey. Some plans provide shelter or nutrition for predators (Heil et al., 2001; Kessler and Baldwin, 2001; Roda et al., 2001). Moreover, plants developed the properties to tolerate certain degrees of tissue damage without their reproductive ability suffering (Strauss and Agrawal, 1999). This is achieved by different mechanisms that all require modifications of growth and resource allocation and is found in different plant species.

All resistance traits are dependent on reorganization of metabolism. Generally, metabolism is separated into "primary" and "secondary" metabolism, where the first is thought to be responsible for growth and reproduction of an unstressed plant, and the latter to be necessary for specific responses to environmental stresses. Traditionally, genes are annotated to either primary or secondary metabolism. This can be misleading, because in recent years, several genes and metabolites of primary metabolism have been found to have secondary functions (Manuscript III). For example, some primary metabolites can act defensively or are involved in defense signalling. Primary metabolism is generally reorganized when resources, which are normally used for growth and reproduction, are diverted to resistance metabolism.

In this thesis, it was studied and discussed, which role primary metabolism plays in plant resistance against herbivory. Using reverse genetics, I investigated the function of a gene, which is annotated to primary metabolism, in plant-insect interactions of the annual tobacco *Nicotiana attenuata* and its specialist herbivore *Manduca sexta*. GAL83, the β-subunit of the SNF1-related protein kinase, was found to be responsible for tolerance towards the herbivore *M. sexta*, in coordination with induced defenses. Moreover, by studying the effects of transformation on plant fitness and several resistance traits, I examined the reliability of the technique of reverse genetics for the study of ecologically relevant genes. I

also discussed the role of primary metabolism in plant resistance to herbivores from a general view.

1.3 The ecology of *N. attenuata* - a model system

The plant used in this thesis is *Nicotiana attenuata* (synonymous with *Nicotiana torreyana* Torr. ex Wats, Solanaceae), an annual plant native in the Great Basin Desert in North-America (Fig. 1). It has adapted to an ecological niche, the immediate post-fire environment after burns of desert vegetation, which are ignited by lightning (Baldwin, 2001). Seeds of *N. attenuata* can rest dormant in the soil for up to 150 years until the next burn occurs. Secondary metabolites from living vegetation, which are washed into the soil by rains, prolong dormancy of the seeds, which chemically eavesdrop on their environment (Krock et al., 2002). When seeds sense an increase of specific combustion compounds from burned plant material, they start to germinate immediately (Schwachtje and Baldwin, 2004).

Fig. 1. *Nicotiana attenuata* and its specialist herbivore *Manduca sexta* in the wilderness of Utah, USA. Photograph kindly provided by Danny Kessler.

This strategy has several advances. After a burn, *N. attenuata* profits from high nutrient levels in the soil, nitrogen, in particular, which is essential for a plant that bases a part of its defenses on nitrogen-demanding compounds, such as nicotine and proteinase inhibitors. A fire eliminates a great portion of plants that potentially compete for soil nutrients, giving *N.*

attenuata the chance to pioneer burned areas and to access high levels of nutrients. Since fires occur irregularly, the appearance of *N. attenuata* is unpredictable, which allows *N. attenuata* to escape herbivores in time. Nevertheless, a specialist herbivore, the tobacco hornworm *Manduca sexta* (Lepidoptera, Sphingidae) has adapted to *N. attenuata´s* major defense alkaloid, nicotine and regularly damages this herb (Steppuhn et al., 2004). Adult moths of *M. sexta* oviposit eggs on leaves of *N. attenuata*, mostly one per plant, and after several days, larvae hatch and begin to feed on the plant's foliage (Kessler and Baldwin, 2002). After ca. 3 weeks larvae are fully grown and can completely defoliate several plants, including buds and flowers. After larvae have molted four to five times they pupate in the soil and hatch.

The ecological model systems *N. attenuata* and *M. sexta* provide an excellent tool with which to study ecological interactions between two native species.

1.4 Tolerance of herbivory

For a long time it has been observed that grasses and other plants, which experience regular foraging, show the ability to recover after herbivore damage. In some cases, even an overcompensational growth has been described, leading to increased fitness of damaged plants compared to undamaged plants. Compensational growth after damage is termed tolerance and can partially, fully or over-compensate for herbivore damage, whereas defenses reduce or prevent the damage itself (Fig. 2). The concept of tolerance initially gained rather theoretical interest, since it turned out to be very challenging to prove evidence of tolerance on an empirical basis (Tiffin, 2000). Even though the availability of sophisticated methods means more studies are being carried out, today the investigation of tolerance is a relatively under-represented field in plant ecology, if compared with research on defenses.

Tolerance can be achieved by different mechanisms in different plant species. Generally, two views of the genetic basis of tolerance can be found in the literature. A majority of researchers believes that tolerance is a result of a general genetic adaptation of the plant, based on numerous quantitative trait loci (QTLs) that influence the capacity of regrowth after damage and affect various aspects of metabolism. In contrast, other researchers view tolerance as an established trait that is based on the action of a few genes, which are regulated after herbivore damage (for review, see Strauss and Agrawal, 1999; Stowe et al., 2000) . These two contradictory views of tolerance have confounded the interpretation of the genetic basis of tolerance, especially because so far no single gene could be directly related to

tolerance. In this study however, this was possible and therefore, the second view of tolerance is supported (Manuscript I).

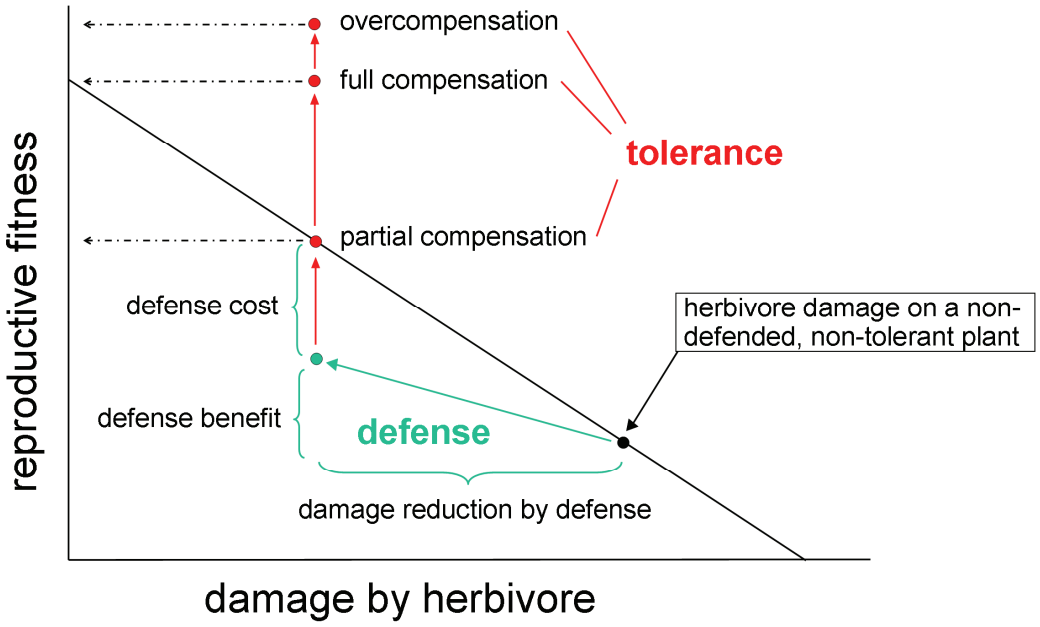


Fig. 2. Relation between tolerance, defense and reproductive fitness of a plant in response to herbivore damage. While defenses reduce the damage caused by a herbivore, fitness may be reduced due to costs of defenses (i.e. resources that are used for production of defenses can not be invested in reproduction). In contrast, tolerance allows a plant to compensate for negative effects of damage without reducing the damage. A combination of defense and tolerance traits in plants may be likely.

1.4.1 Mechanisms of tolerance – primary metabolism is important

Different mechanisms are supposed to underlie compensational responses of plants to herbivory. In general, altered resource allocation and altered growth patterns are supposed to be responsible for tolerance. For example, in some species, an increase of photosynthesis after herbivory has been observed (Cullen et al., 2006); that would lead to increased fixation of carbon, however, it is not a general response of attacked plants. Other plants show no increased photosynthesis or even a decrease (Tiffin, 2000), thus, a direct correlation between altered photosynthesis and tolerance could rarely be shown yet. Because an increase in photosynthetic capacity could also be caused by an increased demand for energy and resources to produce defensive compounds, it is difficult to separate, where and for what additional carbon and energy are used. Another possible mechanism for tolerance is the activation of dormant meristems after damage of vegetative or floral meristems (Bergelson et al., 1996; Mabry and Wayne, 1997). This compensation by growth may only be an advantage

to plants growing in nutrient-rich environments, because additional meristematic growth may compete with already growing flowers and seeds for limited resources. For some plant species, activation of dormant meristems might at least partially be involved in tolerance.

The re-utilization of storage reserves, for example in root tissues, has also been investigated. Such a strategy would be possible for biennial or perennial species that normally accumulate reasonable amounts of reserves during their growing season for later growth in short-day periods. However, a correlation between storage reserves and tolerance remains to be proved. In my thesis, I studied the role of SnRK protein kinases in allocation of resources in the annual plant *N. attenuata,* a plant that normally does not accumulate large storage reserves in roots during development.

1.5 SNF1-related protein kinases (SnRKs) - regulators of primary metabolism in plants

SnRK protein kinases in plants make up a family of kinases, which is related to calcium-dependent protein kinases (CDPK), the largest kinase family in plants. SnRKs have different numbers of members in different plants; in *Arabidopsis* for example there are 38 known, separated in subfamilies SnRK1, 2, and 3 (Hrabak et al., 2003). SnRKs are related to AMPKs (in mammals) and SNF1 (sucrose non-fermenting 1 in yeast), which has recently turned out to be major regulators of primary carbon metabolism, targeting a variety of primary enzymes (Fig. 2). In yeast, under glucose starvation, SNF1 kinases de-repress a variety of glucose-repressed genes, activating alternative pathways for energy production, which are based on nutrients other than glucose. Moreover, SNF1 kinases regulate activity of glycogen synthase, which is responsible for the synthesis of glycogen from excess UDP-glucose, and acetyl-CoA carboxylase, which regulates the first step in plastidic fatty acid synthesis, the formation of malonyl-CoA out of acetyl-CoA. Similarly, AMPKs in mammals are activated under low energy conditions, triggered by a high AMP/ATP ratio, in order to suppress several ATP-consuming pathways (Halford et al., 2003).

SnRKs in plants are composed of three subunits, similar to yeast SNF1 kinases, α: SNF1, β: GAL83/SPI1/SIP2, and γ: SNF4. SNF1 is the catalytical subunit necessary for phosphorylation and is deactivated by autoinhibition. SNF4 releases autoinhibition when it attaches to SNF1 (Fig. 2). The three alternative subunits GAL83/SIP1/SIP2 function as scaffolds and direct the kinase complex to target enzymes or to subcellular locations; for example, GAL83 accumulates in the nucleus (Vincent et al., 2001). The role of SnRKs in plants is only poorly understood. It is now clear that SnRK1 kinases are involved in

deactivating of several enzymes of the primary metabolism by phosphorylation, including nitrate reductase, sucrose phosphate synthase and HMG-CoA reductase. The functions of SnRK2 and SnRK3 kinases in plants may be more diverse and at different stages of development; however, only little is known about these subfamilies (Hrabak et al., 2003). It is supposed that invertases may also be targets of SnRK1s (Halford et al., 2003). SnRK1s can also regulate gene expression on a transcriptional level. For example, they can activate sucrose synthase and a-amylase genes. It is hypothesized that SnRKs are activated in response to high sucrose/low glucose levels, involving one or more upstream kinases. From these findings, it appears that SnRK kinases are involved in the central regulation of carbon metabolism and that they trigger major pathways of primary metabolism. However, a comprehensive understanding of their influence on major alterations of the primary metabolism network and which stresses cause which responses remains to be established.

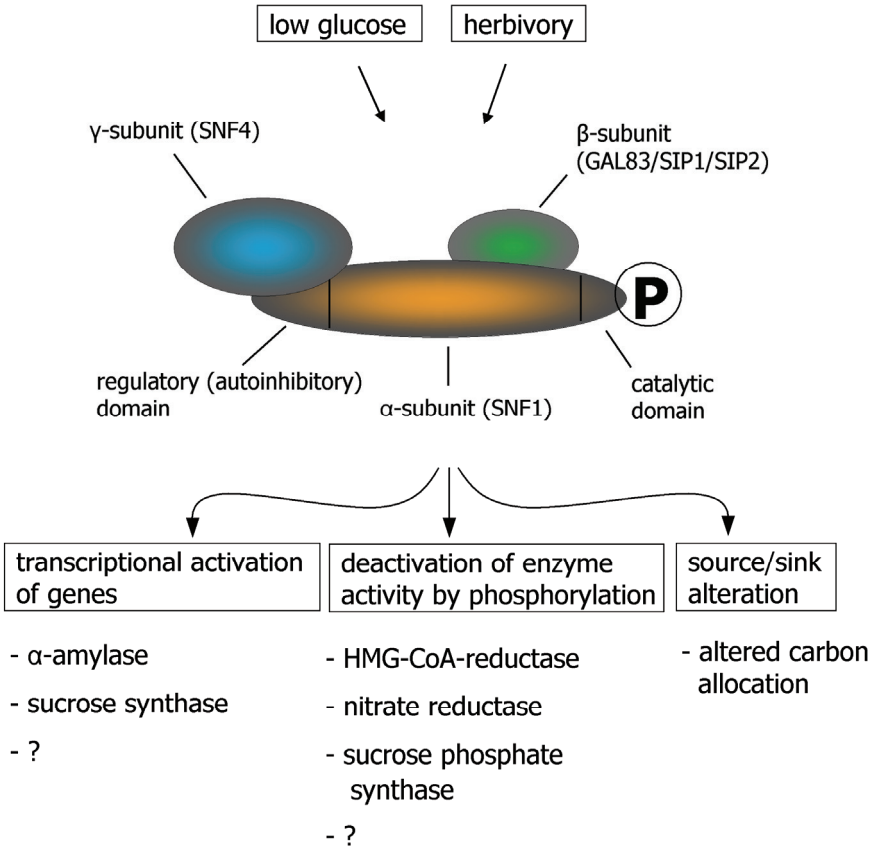


Fig. 3. Structure and function of the SnRK1 kinase complex in plants (modified after Halford et al. 2003).

1.6 Using gene silencing in ecology – the study of gene function

Since the first stably transformed plants were generated in the mid 1980s, transformation has become a widely used tool in plant research. A commonly used technique is gene silencing, called reverse genetics, where specific genes, or even gene families, are precisely silenced. This allows the functional study of a gene *in vivo*, and is increasingly used to study ecologically relevant plant traits. But even though gene silencing has been used for two decades, the underlying molecular mechanisms have been unravelled only partially in the recent years and are still not fully understood.

The principle of the silencing mechanism, RNA interference (RNAi), is based on the presence of double-stranded RNA (dsRNA) molecules in the nucleus (Fig. 3). The natural function of RNA interference is defense against viruses. Once dsRNA molecules are detected, an endonuclease (dicer) cuts them into 21-25 bp fragments, so-called short-interfering RNA (siRNA), which attaches to a RNAi silencing complex (RISC). The RISC protein then can target any single-stranded mRNA molecules which contain a sequence homologue to the siRNA and degrade them. Moreover, the RISC protein unwinds siRNA to produce short single RNA strands, which serve as a template for RNA-dependent RNA polymerases (RdRs). RdRs then generate numerous dsRNA molecules, which amplify the RISC-mediated mRNA silencing. Moreover, double-stranded RNA fragments can regulate DNA methylation (Wassenegger et al., 1994; Matzke and Birchler, 2005).

The idea of gene silencing is to generate dsRNA homologue to the mRNA of the gene that should be silenced. This is carried out by inserting anti-sense fragments of the gene of interest into the plant genome. The transcribed fragments bind to native mRNA and build dsRNA, which activates RNAi. Another possibility is to insert inverted-repeat constructs, which has the advantage that the constructs contain a sense fragment and an anti-sense fragment that bind together to become dsRNA immediately after transcription. This activates RNAi more effectively than antisense fragments that only generate dsRNA when they meet native mRNA in the nucleus, a process which is based on random events. Recently, RNAi has been induced by directly applying dsRNA into the organism, a technique used for transient gene silencing.

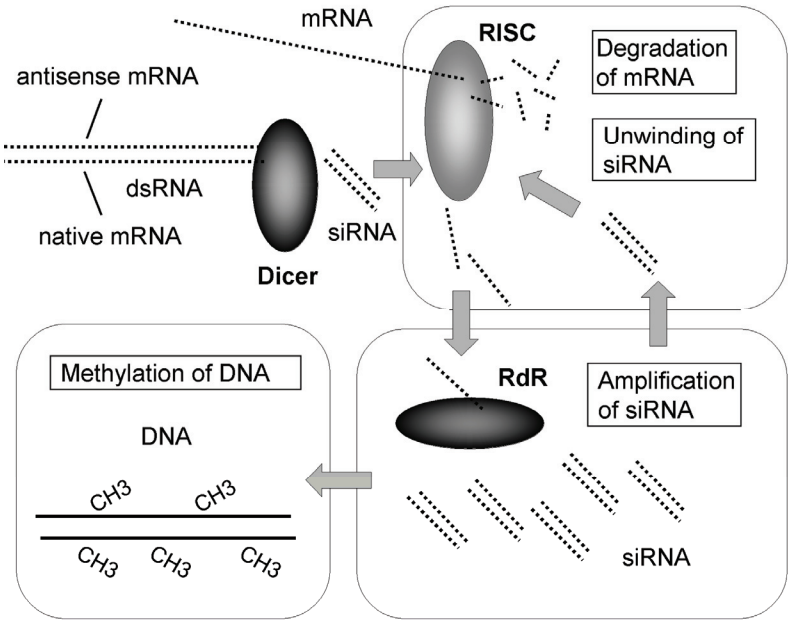


Fig. 4. Mechanisms of gene silencing by RNAi. For details see text.

Today, transformation of plants is mainly carried out by infecting plant tissue with the bacterium *Agrobacterium tumefaciens* that normally causes crown gall disease (Tzfira and Citovsky, 2006). Successful transformation has been established for numerous plant species, even though the molecular basis of the transformation process is only partially unravelled. Besides 'floral dip', a process in which flowers are incubated in a solution containing *A. tumefaciens*, and then produce transformed seeds, seeds, whole seedlings, or cell tissue cultures can also be inoculated.

For transformation, a disarmed strain of *A. tumefaciens*, which does not cause disease symptoms, is used; this strain integrates a defined DNA fragment into the plant genome (transferred DNA, T-DNA) (Gelvin, 2003). The T-DNA is located on a plasmid (binary vector) resident in *Agrobacterium* and is exported from the bacterium and integrated into the plant genome by several virulence (*vir*) genes located on a Ti (tumor-inducing) plasmid. A single-stranded (ss) T-DNA molecule is generated by the vir proteins and transferred into the host cell through a type IV secretion system, also established by vir proteins. The ssDNA is protected by vir proteins and directed to the nucleus by using the intracellular transport machinery; the cytoskeleton is used as a track. It is believed that vir proteins do not have the necessary properties to integrate the T-DNA into the genome. The vir protein-ssDNA complex is thought to use various cellular mechanisms of the host cell (Tzfira and Citovsky, 2006), such as the nuclear import and transcription machinery. It appears that the T-DNA is inserted at random places in the genome, slightly less biased at the centromer regions, probably because genome activity there is relatively low.

A disadvantage of the integration of T-DNA by *Agrobacterium* is that it can cause a variety of alterations in the host genome. Among these are chromosomal rearrangements, insertions of superfluous DNA or DNA repeats of different length; deletion of DNA; multiple T-DNA insertions; interruptions of genes when T-DNA is inserted into coding sequences or introns; or pleiotropic effects caused by the new DNA. Another source for genomic change is tissue culture, which is required for transformation of some plant species and depends on the application of growth promoting substances. This may cause epigenetic and heritable (somaclonal) genetic variation (Larkin and Scowcroft, 1981; Kaeppler et al., 2000; Cellini et al., 2004; Filipecki and Malepszy, 2006; Latham et al., 2006).

These molecular side-effects of transformation and tissue culture may take place regularly, but to different degrees; therefore, it is difficult to estimate their frequency and the consequences for the plant phenotype. Apart from detailed studies on the molecular level, what impact those side-effects could have on plant phenotypes has been rarely studied. Therefore, when gene silencing is used in ecological gene function studies, it is necessary to determine whether a specific transformation procedure may induce unintended effects, and, in the worst case, may obfuscate the study of the effects of silencing of a specific gene. An optimal way to test a transformation procedure for unintended effects is to use so-called empty vector controls. These are plants which have undergone all steps of a transformation and carry a T-DNA fragment. The T-DNA does not contain any silencing information, but it does contain every other information needed for gene silencing, including a promoter, a terminator, and an antibiotic resistance gene, which is needed for the screen of homozygous transformants. Empty vector controls are only sometimes included in studies that use reverse genetics and are studied here to evaluate the transformation of *N. attenuata*.

2. List of manuscripts: Contents and author contributions

Manuscript I

SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots.

Jens Schwachtje, Peter E. H. Minchin, Siegfried Jahnke, Joost T. van Dongen, Ursula Schittko, Ian T. Baldwin

Proceedings of The National Academy of Sciences of the USA (2006), 103 (34), 12935-12940

This manuscript describes the function of a subunit of a SNF1-related kinase, GAL83, for tolerance of herbivory. GAL83 is rapidly downregulated in *Nicotiana attenuata* in response to oral secretions of the tobacco hornworm *Manduca sexta*. Shortly after simulated herbivory by *M. sexta*, wild type plants show reduced free sugars (glucose, fructose, sucrose) in sink and source leaves, increased invertase activity in roots, and an increased allocation of carbon to roots, what could be shown by tracking of ^{11}C-labelled CO_2 that was applied to source leaves. Moreover, plants silenced for GAL83 (asGAL83) by RNAi show an increased allocation of carbon to roots compared to wild type plants when plants are not induced by oral secretions. By measuring fitness parameters (flowers, height, aboveground biomass and seed capsule production) it could be shown that asGAL83 plants invest their increased root resources in reproduction and, as a result, show delayed senescence after simulated herbivory. These results demonstrate that GAL83 regulates carbon allocation in plants so that they can tolerate herbivory.

Under the supervision of Ian T. Baldwin I planned and realized all experiments. Siegfried Jahnke and Peter E. H. Minchin helped setting up the $^{11}CO_2$ measurements at the Phytosphäre at the FZ Jülich and Peter E. H. Minchin carried out calculation of the raw data and helped interpret results. Joost T. van Dongen helped me measuring free sugars and enzyme activities at the MPI for Molecular Plant Physiology in Golm. Ursula Schittko carried out Northern blotting to study the

transcriptional regulation of GAL83. I wrote the first draft of the manuscript and refined it with Ian T. Baldwin for the final version.

Manuscript II

Reverse genetics in ecology.

Jens Schwachtje, Susann Kutschbach, Ian T. Baldwin

Accepted by PLoS ONE

In this manuscript the technique of gene silencing - reverse genetics - was critically evaluated with respect to the study of ecological gene functions *in vivo*. To test, whether plant transformation with tissue culturing and *Agrobacterium* infection results in unintended side-effects on the genome that may obfuscate the study of the function of a specific gene, 5 independently transformed plant lines that carry the T-DNA that is necessary for transformation but no gene silencing information (empty vector controls, EVC), were compared to wild types of *Nicotiana attenuata* (Solanaceae) for a variety of critical ecological parameters in response to simulated herbivory. For all measured parameters, nicotine production (a defense), trypsin proteinase inhibitor activity (a defense), levels of the plant defense hormone jasmonic acid (JA) and its conjugate JA-Isoleucine, growth, seed capsule production (the crucial parameter for Darwinian fitness), and the transcription of 1,400 resistance- and primary metabolism-related genes (measured with microarrays), no statistically significant differences between the 5 EVC lines and wild types could be measured. A statistical power analysis revealed that that it takes 3 orders of magnitude more replicates to detect significant fitness differences between EVC lines and wild types than between wild types and an irPI line that is silenced for a proteinase inhibitor gene and produced significantly more capsules than wild types.

This study demonstrated that *Agrobacterium*-mediated transformation of *N. attenuata* does not alter the phenotype in an unintended manner, making reverse genetics a reliable tool for the study for ecological gene function *in vivo*.

Under the supervision of Ian T. Baldwin I planned and realized all experiments and evaluated the data. Susan Kutschbach helped carrying out microarray

studies, fitness evaluation and measurement of defense traits (nicotine and proteinase inhibitors). I wrote the first draft of the manuscript and refined it with Ian T. Baldwin for the final version.

Manuscript III

Why does herbivore attack reconfigure primary metabolism?

Jens Schwachtje, Ian T. Baldwin

Accepted by Plant Physiology, invited review

In this review, recent findings of the functions of primary metabolism for plant responses to herbivory are described and the role of primary metabolism for plant resistance is discussed from a whole-organism perspective. New unbiased approaches, which characterize transcriptomic, metabolomic, and proteomic changes in herbivore-attacked plants, have challenged the view that "secondary" metabolism functions to meet environmental challenges, and that "primary" metabolism supports growth. The hundreds of genes regulated during the plant-herbivore or -pathogen interaction have been analyzed with microarray studies, and almost all aspects of metabolism are represented, with a substantial fraction coming from primary metabolism.

In this review, 4 overlapping functional explanations for this reconfiguration are discussed:

I. Resistance traits are costly and frequently up-regulated after attack, requiring reductions in growth, reproduction or storage and/or increases in assimilation to meet their metabolic demands. These changes in resource allocation can be either *acute*, driven by immediate reductions in resources, or *anticipatory*, occuring before resource supply limits defense activation.
II. Rather than supporting defense responses, reconfiguration can support the physiological adjustments plants must make to tolerate herbivory and reduce the negative fitness consequences of herbivore attack.
III. Primary metabolites can function as signals in defense pathways.

IV. Induced changes in primary metabolism can themselves be defensive.

This review resulted from extensive discussions about the function of primary metabolism with Ian T. Baldwin. I wrote the review with the help of Ian T. Baldwin.

SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots

Jens Schwachtje*, Peter E. H. Minchin†‡, Sigfried Jahnke†, Joost T. van Dongen§, Ursula Schittko¶, and Ian T. Baldwin*‖

*Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany; †ICG-III Phytosphäre, Forschungszentrum Jülich, 52425 Jülich, Germany; §Department of Metabolic Networks, Max Planck Institute for Molecular Plant Physiology, 14476 Potsdam, Germany; and ¶Department of Biology, Minot State University, Minot, ND 58707

Edited by May R. Berenbaum, University of Illinois at Urbana–Champaign, Urbana, IL, and approved July 3, 2006 (received for review March 21, 2006)

Herbivore attack elicits costly defenses that are known to decrease plant fitness by using resources that are normally slated for growth and reproduction. Additionally, plants have evolved mechanisms for tolerating attack, which are not understood on a molecular level. Using ^{11}C-photosynthate labeling as well as sugar and enzyme measurements, we found rapid changes in sink–source relations in the annual *Nicotiana attenuata* after simulated herbivore attacks, which increased the allocation of sugars to roots. This herbivore-induced response is regulated by the β-subunit of an SnRK1 (SNF1-related kinase) protein kinase, GAL83, transcripts of which are rapidly down-regulated in source leaves after herbivore attack and, when silenced, increase assimilate transport to roots. This C diversion response is activated by herbivore-specific elicitors and is independent of jasmonate signaling, which regulates most of the plant's defense responses. Herbivore attack during early stages of development increases root reserves, which, in turn, delays senescence and prolongs flowering. That attacked GAL83-silenced plants use their enhanced root reserves to prolong reproduction demonstrates that SnRK1 alters resource allocation so that plants better tolerate herbivory. This tolerance mechanism complements the likely defensive value of diverting resources to a less vulnerable location within the plant.

carbon-11 | defense | plant–herbivore interactions | tolerance

Plants have evolved a variety of mechanisms for reducing the negative impact of herbivore attack on fitness; these mechanisms include direct and indirect defenses and tolerance (1). Defenses are costly, expending energy and resources that could otherwise be used to grow and generate offspring. Inducible defenses allow plants to invest resources into defense only when needed. Although defenses limit the extent of damage, even well defended plants lose large amounts of tissue when attacked by herbivores that have adapted to their defenses. Then, plants would benefit from tolerance, which minimizes the fitness consequences of tissue loss to herbivores (2–4). Defense against, and tolerance of, herbivory are not mutually exclusive; most plant–insect interactions likely combine both (5, 6). In contrast to the rapid advances in our understanding of defense mechanisms, little is known about the traits that allow plants to tolerate herbivore damage.

Tolerance, which is measured by comparing the fitness of a genotype in environments with and without attackers, remains uncharacterized at the molecular level (2, 7). At a physiological level, increases in photosynthetic rate, branching, and storage in belowground tissues are thought to be involved (8–10). These responses require the tuning of primary metabolism, for which mutant screens and other reverse genetic approaches with model plants have yet to yield molecular regulators. Host plants that have coevolved with adapted herbivores likely have elaborate defense and tolerance responses to minimize the fitness consequences of herbivory.

The postfire annual of the Great Basin Desert of the United States, *Nicotiana attenuata* Torr. ex Wats. (Solanaceae), copes with a variety of herbivores from different feeding guilds by dramatically up-regulating and tailoring the expression of a variety of defenses to particular attackers (11). For example, the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) has evolved resistance to nicotine (12), the plant's major defense alkaloid. The plant recognizes attack from *M. sexta* larvae when fatty acid–amino acid conjugates (FACs) from larval oral secretions and regurgitants (Rs) are introduced into the wounds during feeding, which down-regulates nicotine production and up-regulates a suite of other direct and indirect defense responses, all requiring jasmonate (JA) signaling for their activation (13–15). Despite these defense responses, *M. sexta* larvae regularly defoliate *N. attenuata* plants in native North American populations and are responsible for most of the leaf damage in these populations (16, 17). Therefore, we predict that *N. attenuata* benefits from tolerance traits to complement its elaborate defense responses and that tolerance results from altered resource allocation (3) that is closely coordinated with herbivore attack.

Results and Discussion

^{11}C Labeling Reveals C Partitioning to Roots. Because defense elicitation of *N. attenuata* occurs rapidly [transcriptional and metabolic responses start within minutes of attack (14, 18)], we measured C partitioning between shoot and root to estimate changes in resource allocation shortly after herbivore attack. We used $^{11}CO_2$, a short-lived C isotope with a half-life of 20.4 min (<2% of initial activity after 2 h), which allows for *in vivo* tracking of photoassimilate partitioning with several measurements per plant per day (19). Partitioning was measured both before and after elicitation in the same plant in real time. We supplied $^{11}CO_2$ to source leaves of young rosette-stage WT plants. To elicit a strong and reproducible response to *M. sexta* attack, we wounded three source leaves (Fig. 1*A*) with a fabric pattern wheel twice in 3 h and immediately applied R to the wounds, a treatment that elicits the same transcriptional and defensive responses as *M. sexta* feeding (20–22).

By providing ^{11}C to source leaves, we were able to measure C partitioning to roots and shoots of each unmanipulated plant (Figs. 1 *B* and *C* and 2). Source leaves were elicited and subsequently supplied for a second time with ^{11}C. By calculating the relative change of root C fractions before (10 a.m.) and after (4 p.m.) treatments, we discovered a significant (10%) increase in C allocation to roots after treatment with R but not when puncture wounds were treated with distilled water (W) (Fig. 2*A*).

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: SnRK1, SNF1-related kinase; FAC, fatty acid–amino acid conjugate; R, regurgitant; JA, jasmonate; W, distilled water; SuSy, sucrose synthase; *as*, antisense.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY460336).

‡Present address: Hort Research, Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand.

‖To whom correspondence should be addressed. E-mail: baldwin@ice.mpg.de.

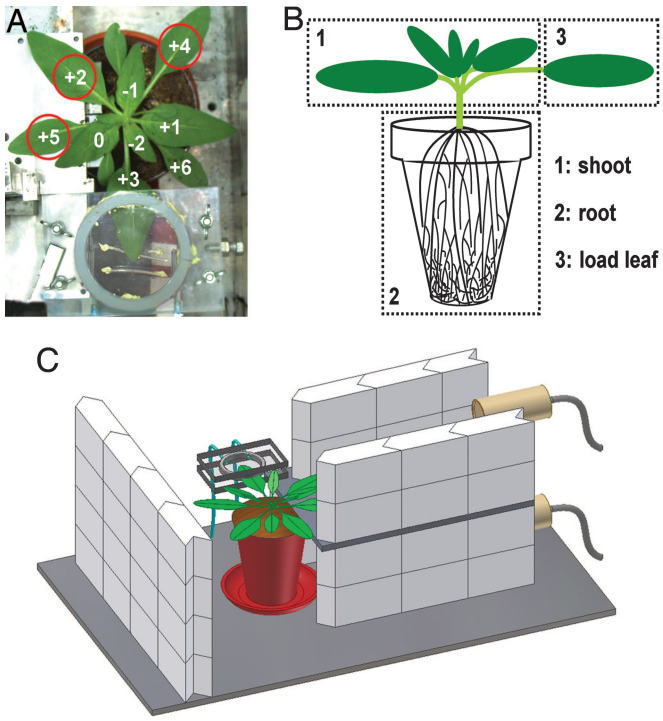


Fig. 1. Experimental setup. (*A*) Numbers denote the mature (source) leaves used for either $^{11}CO_2$ pulse feeding (+3) or elicitation (+2, +4, and +5); immature (sink) leaves are labeled with negative numbers. The sequence indicates the leaf age; the larger the number, the older the leaf. (*B*) Scheme of detection areas. The load leaf was separately measured to control $^{11}CO_2$ pulses. (*C*) Scheme showing the positions of the shoot and root detectors as well as the lead and tungsten shielding (collimation) needed to separate the field of view of the different detectors.

The effect of R was completely reproduced when FACs (*N*-linolenoyl-L-Gln and *N*-linolenoyl-L-Glu), which occur naturally in R and are known to elicit *N. attenuata*'s responses to *M. sexta* attack (13, 21), were added to puncture wounds (Fig. 2*A*). To better understand the magnitude of the R/FAC-elicited changes on C allocation to roots, we completely removed all aboveground sinks by removing the sink leaves and the stem of a 5-cm elongated plant while keeping source leaves intact. This treatment should have caused a dramatic alteration in sink–source balance between shoot and root, but it merely doubled the allocation of C to roots compared with the R/FAC treatment (Fig. 2*A*), demonstrating how strongly R elicitation influenced assimilate partitioning.

Furthermore, W and R treatments were accompanied by significant changes in sugar metabolism 5 h after elicitation. Sucrose transport by the phloem is understood to be a gradient-driven process whereby sucrose is actively loaded by transporters into source tissues and passively unloaded (symplasmically or apoplasmically) into sink tissues. Sink strength, which is partially regulated by sucrose-cleaving enzymes [invertases and sucrose synthase (SuSy)], helps drive the process (23–25). Neither W nor R treatments influenced the activity in leaves of any of the invertases measured (Fig. 3 *A* and *B*) or of SuSy (data not shown).

Only in roots did both treatments strongly increase soluble acid (vacuolar) invertase activity (Fig. 3*C*). This increase in sugar-cleaving activity likely increases the sink strength of roots and facilitates root growth as recently shown by quantitative trait locus and mutant analysis of this invertase in *Arabidopsis thaliana* (26). Because a plant's sink organs compete continuously with each other for photoassimilates, an increase in root sink strength will reduce the amount of photoassimilates transported to shoot sinks. Indeed, the amount of sugars measured in sink leaves of both W- and R-treated plants were strongly reduced (Fig. 3*B*), and R-treated plants had significantly lower sucrose contents in sink leaves than did W-treated plants (Mann–Whitney *U* test, $P = 0.0143$; $n = 5$; Fig. 3*B*). Significantly, sucrose and fructose levels were reduced in source leaves (which represent the major aboveground biomass of rosette-stage plants) in R-treated plants but not in W-treated plants (Fig. 3*A*). This finding indicates that roots of R-treated plants recruit sugars from source leaves much more efficiently than do roots of control- and W-treated plants.

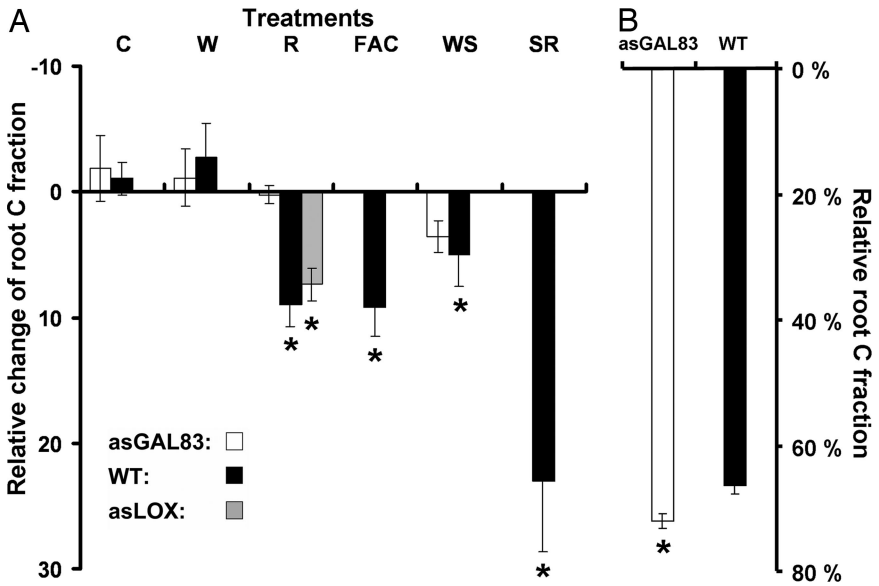


Fig. 2. C allocation in *N. attenuata*. (*A*) Relative change (mean ± SE, $n = 3–6$) of the root-partitioned C fraction of *as*GAL83, WT, and *as*LOX plants after 5 h in response to different types of induction (C, control; W, wounding; R, R elicitation; FAC, application of FACs; WS, wounding of sink leaves; SR, aboveground sink removal) as measured by $^{11}CO_2$ application. Asterisks indicate significant difference from WT C (for each comparison with WT C, Mann–Whitney *U* test, $P < 0.05$). (*B*) Fraction (mean ± SE, *n* WT = 45, *n as*GAL83 = 27) of assimilates partitioned to roots of unelicited plants (Mann–Whitney *U* test, $U < 462.5$, $P = 0.0134$).

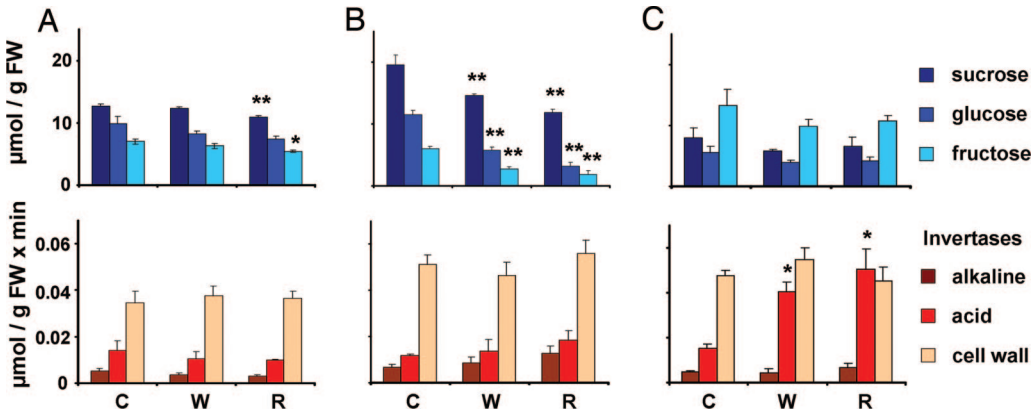


Fig. 3. Enzyme activities of alkaline invertase, soluble acid (vacuolar) invertase and cell wall invertase, and soluble sugar contents (sucrose, glucose, and fructose) of source leaves (*A*), sink leaves (*B*), and roots (*C*) 5 h after W and R treatment (see Fig. 1). (*A*) Mann–Whitney *U* test, sucrose, $U < 25$, $P = 0.009$; fructose, $U < 24$, $P = 0.0163$; $n = 5$. (*B*) Mann–Whitney *U* test, $U < 20$, $P < 0.01$; $n = 5$. (*C*) Mann–Whitney *U* test, wounding, $U < 9$, $P < 0.05$; R elicitation, $U < 9$, $P < 0.05$; $n = 4$. SuSy activities (data not shown) were not changed after any treatment. *, $P < 0.05$; **, $P < 0.01$.

Apparently, additional sugar transporter activity is elicited in roots or source leaves of R-elicited plants compared with those of W-treated plants. The soluble sugar content in roots did not change after R treatment (Fig. 3*C*), and we infer that the extra supply of sugars was rapidly used for respiration, storage carbohydrate metabolism, or growth.

To determine whether the R/FAC-elicited response in C partitioning was mediated by JA signaling, we tested the response of *N. attenuata* plants (*as*LOX) that had been transformed with the endogenous lipoxygenase gene (NaLOX3); this gene supplies lipid hydroperoxide substrates for JA biosynthesis (12) and, when silenced by expression in an antisense (*as*) orientation, highly impairs a plant's defense responses (12). The elicited C partitioning response in these plants did not differ from that in WT plants (Fig. 2*A*), demonstrating that JA signaling is not required and that the rapid increase in C allocation to roots after herbivore elicitation is fully functional in defenseless plants with silenced JA signaling.

GAL83 Mediates C Allocation. To identify the genetic basis of the C partitioning response to *M. sexta* attack, we used a differential display PCR of control and *M. sexta*-attacked *N. attenuata* plants (27). We found that GAL83, a β-subunit of a heterotrimeric SnRK1 (SNF1-related kinase) kinase complex (28), was down-regulated in source leaves, whereas the catalytic α-subunit SNF1 was not (Fig. 4). That down-regulation is rapid (<1 h) (see *Supporting Text*, which is published as supporting information on the PNAS web site) and not elicited by methyl JA, a treatment that strongly elicits defenses (29), makes GAL83 an interesting candidate as a mediator of the resource allocation response.

Collectively, SnRK1s are kinases that function as cellular fuel gauges and play central roles in cell energy metabolism, regulating several key enzymes in sugar metabolism (28, 30, 31). SnRK1s comprise three subunits: α, which is composed of SNF1; three possible β-subunits, SIP1, SIP2, and GAL83; and γ, which is composed of SNF4. Together, these subunits form the active complex. Homologues occur in all kingdoms and are well studied in yeast, where they activate glucose-repressed genes when the cells' energy reserves run low (28, 30), and in mammals, where they are involved in regulating glucose uptake and gluconeogenesis and are also necessary for diabetes therapy (32). In yeast, GAL83 is thought to direct the kinase complex to the nucleus (33); however, its function in plants is not well understood. *as*GAL83 potato plants, for example, show altered root and tuber development (34).

To study the role of GAL83 in the R/FAC-elicited C allocation response, we transformed *N. attenuata* plants to express GAL83 in an *as* orientation. From 20 independently transformed homozygous lines, 6 were screened for transcriptional down-regulation of GAL83 in roots, where it is highly expressed in WT plants (Fig. 7, which is published as supporting information on the PNAS web site). We predicted that *as*GAL83 plants would mimic the C allocation pattern of elicited WT plants if GAL83 transcripts were continuously down-regulated. Two independently transformed single-insert homozygous *as*GAL83 lines were tested and found to have greater root:shoot dry mass ratios compared with WT plants, although total mass remained the same (Fig. 8, which is published as supporting information on the PNAS web site). With the $^{11}CO_2$ technique, we found that a single-insert homozygous line of *as*GAL83 plants that accumulated only 22% of the GAL83 transcripts of WT plants (see *Supporting Text*) transported ≈10% more C to the root than did WT plants (72.0% vs. 66.3%; Fig. 2*B*). R treatment of *as*GAL83 plants did not alter their constitutively increased allocation to roots (Fig. 2*A*). These results clearly demonstrate that the

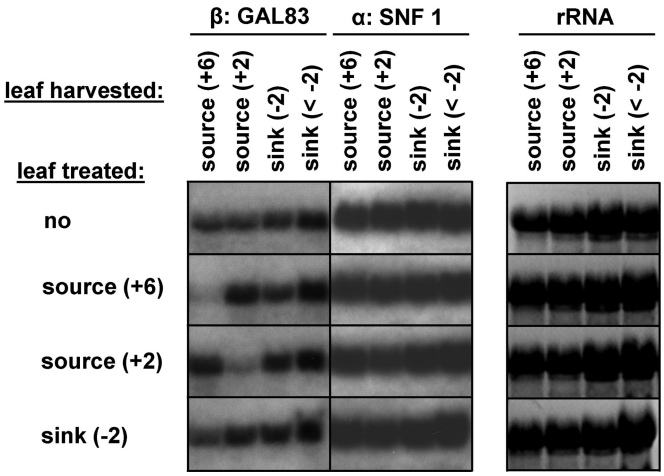


Fig. 4. Northern blot of GAL83 and SNF1 (α-subunit) mRNA expression in *N. attenuata* sink and source leaves (see Fig. 1*A*) 30 min after 4 h of repeated wounding and application of *M. sexta* R. Total RNA was pooled from 27 replicates for each treatment. GAL83 transcripts were down-regulated locally in treated source leaves and slightly down-regulated systemically after treatment of sink leaves. The transcript of the α-subunit, SNF1, was not regulated in leaves. rRNA was used as a loading control.

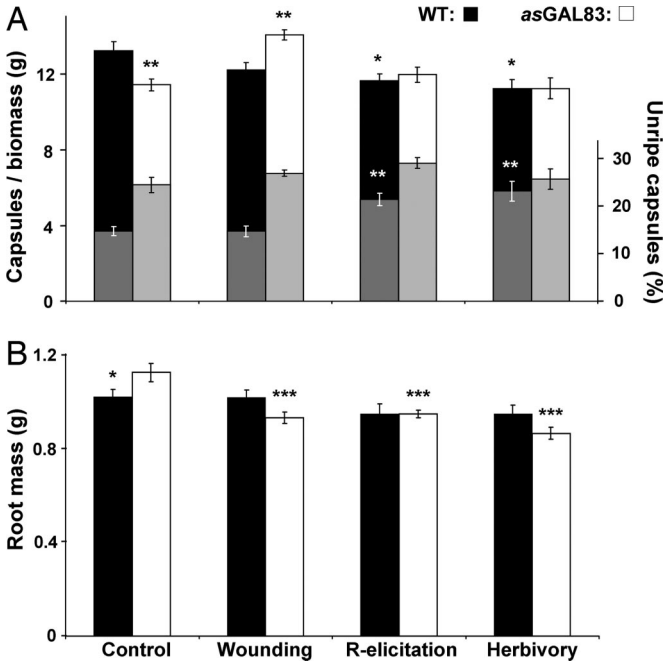


Fig. 5. Seed capsules per g of biomass and percentage of unripe capsules of the total capsules (*A*) and root (dry) masses (*B*) of WT and *as*GAL83 plants 54 days after elicitation. (*A*) Seed capsule number per g of biomass, mean ± SE (percentage of unripe capsules of total capsules is shown by gray bars). Asterisks over capsules/biomass bars (control and wounding) indicate significant differences between lines (Mann–Whitney *U* test, $P < 0.01$). Asterisks over capsules/biomass bars (R elicitation and herbivory) indicate significant differences between treatment and control plants (Mann–Whitney *U* test, $P < 0.01$). Asterisks over unripe capsules bars indicate differences compared with control (Mann–Whitney *U* test, $P < 0.01$). (*B*) Final root mass, mean ± SE. The *as*GAL83 controls do have a significantly larger root mass than WT controls (unpaired *t* test, $DF = 26$, $T = 2.071$, $P < 0.05$). All elicited *as*GAL83 root masses are significantly smaller than those of *as*GAL83 control plants (ANOVA, $F_{3,46} = 15.525$, $P < 0.0001$, post hoc $P < 0.001$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

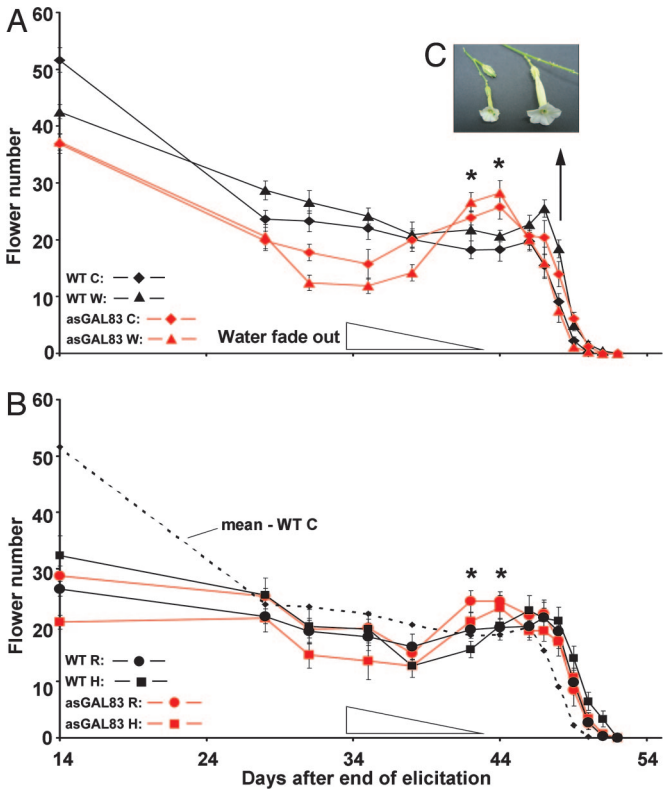


Fig. 6. Flower production of WT and *as*GAL83 plants after elicitation (mean ± SE). (*A*) Control and wounding treatment. (*B*) R elicitation and herbivory. (*A* and *B*) Fully opened flowers were measured. Watering of plants was gradually reduced over a 10-day period (see *Supporting Text*). Asterisks indicate that *as*GAL83 plants produced significantly more flowers than did WT (unpaired *t* test, $t > 3$, $P < 0.05$). (*C*) Late flowers of WT controls (left) are smaller than those of WT H-treated plants (right) on day 49 after elicitation; the same difference was observed for *as*GAL83 flowers.

GAL83 cofactor of the *N. attenuata* SnRK1 complex regulates the allocation of C within the plant in response to herbivore attack and is elicited by FACs of *M. sexta* R.

Root Resources Provide Tolerance. To determine whether *M. sexta*-attacked *N. attenuata* plants realize a fitness benefit from an increase in C allocation to roots, we conducted a long-term greenhouse experiment in which rosette-stage WT and *as*GAL83 plants were grown in 1-liter pots. For 6 days before stalk elongation commenced, we either (*i*) elicited plants with W or R twice per day (at 10 a.m. and 4 p.m.) with two source leaves treated simultaneously so that, each day, four different leaves were treated or (*ii*) allowed four *M. sexta* larvae to feed freely for 6 days on source leaves (a treatment that we call "H"). We monitored stalk height, flower number, and seed capsule production (as correlates of fitness through the male and female function, respectively) (29) for ≈2 months until all plants had senesced and measured final root and shoot biomasses.

GAL83-silenced plants were smaller than WT plants after all treatments (Fig. 9, which is published as supporting information on the PNAS web site) because of increased assimilate allocation to roots and its associated opportunity costs for aboveground growth. Unelicited *as*GAL83 plants (controls) produced significantly fewer capsules per gram of final biomass than did unelicited WT controls (Fig. 5*A*), and, accordingly, root mass at senescence of *as*GAL83 controls was significantly greater than that of WT controls (Fig. 5*B*). Interestingly, W-elicited *as*GAL83 plants produced significantly more capsules related to biomass than did W-treated WT plants, which did not regulate GAL83 (Fig. 5*A*). This compensatory response was associated with a 17% reduction in root mass in comparison with *as*GAL83 controls (Fig. 5*B*). Furthermore, root masses of *as*GAL83 plants were significantly reduced after all treatments (Fig. 5*B*). These results demonstrate that GAL83 regulates resource storage in the roots; these resources can be mobilized to support seed production, the principal fitness "currency" of this annual plant. Moreover, leaf damage during rosette-stage growth among all genotypes appears to allow a plant to use root resources more effectively during reproduction (Fig. 5*A*) by unknown mechanisms that deserve additional attention.

Watering was reduced over a 10-day period after plants had attained maximum stalk heights to simulate the normal soil-drying regime that these plants experience in their native habitat (see *Supporting Text*). During this period of decreased water availability, flower production in *as*GAL83 plants increased significantly more than in WT plants (Fig. 6 *A* and *B*). In nature, soil desiccation appears to function as an (abiotic) signal that plants use to mobilize their remaining root storage for a final reproductive effort before completely senescing. At the final harvest of the experiment, which was conducted when flowering had ended, *as*GAL83 plants had significantly more unripe capsules relative to all capsules than did WT plants (24.51% ± 1.6% vs. 14.76% ± 0.9%; Fig. 5*A*), reflecting their larger final flowering effort, which in turn was likely fueled by their larger

root reserves. Flowers mature into ripe capsules 10–12 days after pollination.

Within-genotype comparisons showed that R and H treatments resulted in reduced growth and significantly fewer capsules in each genotype (Figs. 9 and 10, which are published as supporting information on the PNAS web site), as well as a significant reduction of capsules related to biomass (Fig. 5*A*), which likely reflects the fitness costs of the elicited defense responses. In this species, R-elicited defensive trypsin proteinase inhibitor production is known to decrease stalk height and reduce capsule production (35, 36). However, WT flowering was significantly prolonged (by 2–3 days) by herbivore elicitation, to the extent that the number of flowers produced in the last week was 1.67-fold greater than in unelicited controls (Fig. 6 *A* and *B*). Consequently, R- and H-elicited WT plants produced significantly more unripe capsules than did wounded WT plants, which did not regulate GAL83 (unpaired *t* test, R elicitation: $D = 26$, $t = 2.083$, $P < 0.05$; herbivory: $D = 22$, $t = 2.148$, $P < 0.05$; data not shown), reflecting their increased use of reserves for the final flowering effort (Fig. 6). As a result, the proportion of unripe capsules of all capsules produced by WT plants significantly increased after R and H treatment (Fig. 5*B*), indicating a shift of resource investment into reproduction to a later stage of development. The delayed senescence of elicited plants correlated with their larger root reserves, which likely provided the resources required for the final reproductive effort.

In contrast to the plants in the R and H treatments, W-treated plants experienced no reduction of flower production or capsule number (absolute or related to biomass) compared with unwounded plants (Figs. 5*A*, 6, and 10). Although wounding elicited some defenses, such as nicotine production, and resulted in the same amount of leaf damage experienced by R-treated plants, wounded plants were able to fully compensate for the associated costs. Wounding was accompanied by an increase in lateral branching (unpaired *t* test, WT: $t = -4.547$, $P < 0.0001$; *as*GAL83: $t = -2.657$, $P = 0.0144$), which was not observed in R- and H-treated plants (unpaired *t* test, WT R: $t = -1.402$, $P = 0.15$; WT H: $t = -0.655$, $P = 0.52$; *as*GAL83 R: $t = -1.742$, $P = 0.11$; *as*GAL83 H: $t = -1.465$, $P = 0.16$), suggesting that a plant's regrowth response to wounding is altered when the elicitors of insect herbivores are introduced into wounds.

Tolerance and Its Potential Application. All plants allocate resources among traits that function in growth, reproduction, and defense to optimize their chances of being represented in future generations. Tolerance may be the best strategy for a plant to extricate itself from cycles of defensive escalation with its adapted herbivores. When attacked by adapted herbivores, host plants are likely to combine defense and tolerance responses, yet how these responses are integrated has been unknown until now. When attacked by the nicotine-adapted *Manduca* larvae, *N. attenuata* tunes its repertoire of induced defenses for maximal effectiveness (37–41) but also begins to bunker recently fixed C in its roots. Because root tissue is safe from this folivore, C stored there may be a means of immediately removing it from harm's way. Once allocated to the roots, the C can be used to sustain seed production at the end of the plant's life, after the *Manduca* larvae have pupated. How GAL83, the β-subunit of the plant's SnRK1, mediates this C hoarding behavior remains unknown. The same elicitors (FACs) that activate the tuning of the induced defense responses after *M. sexta* attack activate this C storage in the roots, albeit by a different signal transduction cascade. By reconfiguring resource allocation, the plant gains a measure of tolerance to this voracious herbivore. That attacked GAL83-silenced plants use their enhanced root reserves to prolong reproduction demonstrates that SnRK1 supports a plant's tolerance to herbivory and that single genes can have large effects, contrary to the assumptions of previous quantitative genetic analyses (2, 42, 43).

The success of agriculture is based on breeding strategies that target the allocation of resources to harvestable parts (tubers, stems, fruit, etc.). The discovery that FACs trigger GAL83 extends to breeders the ability to alter the allocation of assimilates to different sink tissues (roots) by means of a simple environmental cue, which could represent a major biotechnological breakthrough.

Methods

For plant growth, treatment details, transformation, and RNA extraction and determination, see *Supporting Text*.

Plant Fitness Measurements. Stalk elongation, branch length, flowering, seed capsule production, and final root and shoot mass of all plants were measured as fitness determinants. To compare the lifetime reproductive performance among genotypes and treatments, we recorded for each plant: (*i*) stalk length starting on the day with measurable stalk growth (14 days after transplanting, when elicitation was finished) for 33 subsequent days, (*ii*) flower numbers (fully opened flowers) from day 14 after elicitation until end of flowering (52 days after elicitation), (*iii*) the number of ripe and unripe seed capsules 54 days after elicitation (the number of capsules per plant reflects the lifetime reproductive output in *N. attenuata* under natural or glasshouse conditions), and (*iv*) final root and shoot mass when capsule production had ended.

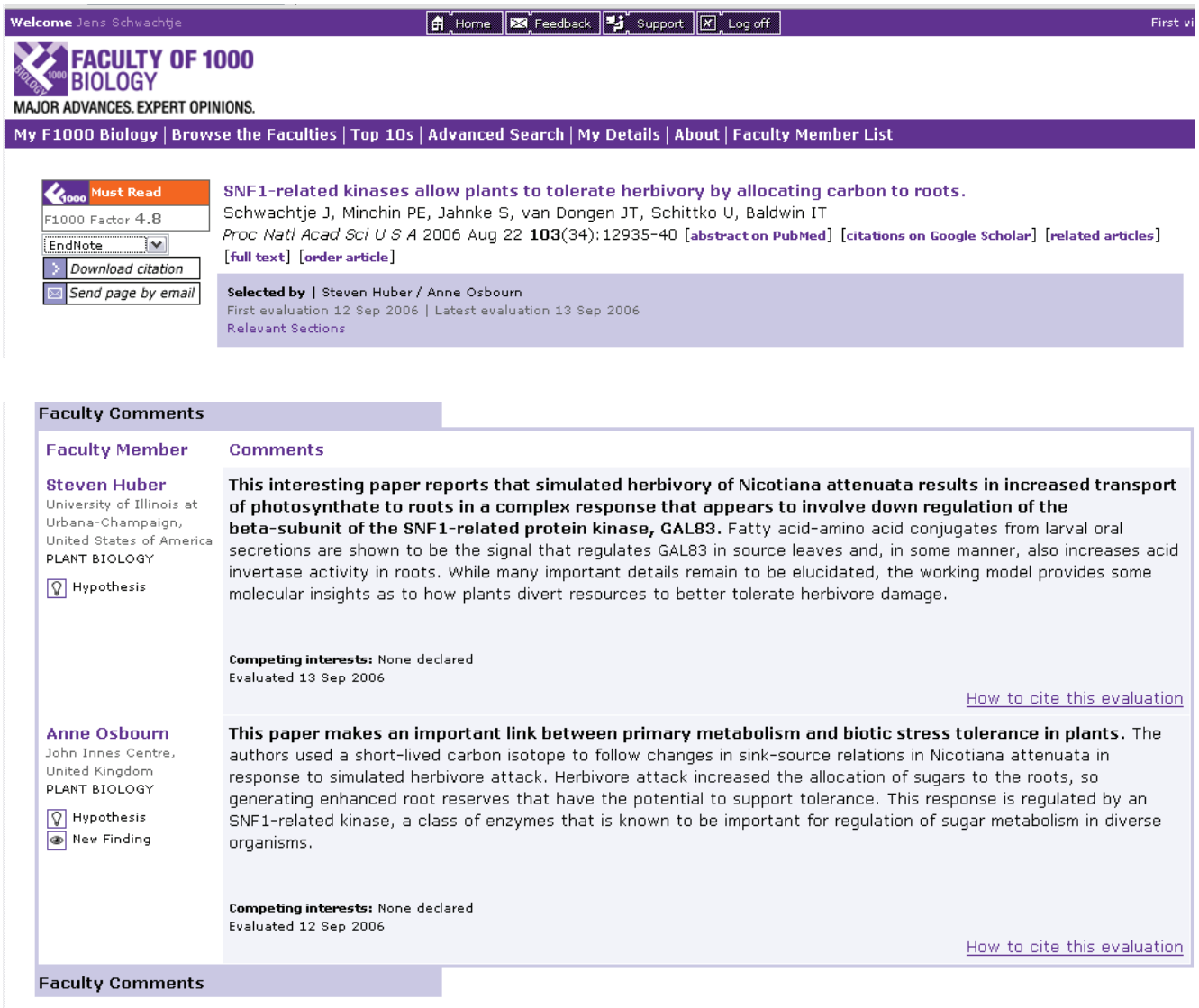
^{11}C Measurements. ^{11}C measurements were carried out at the Phytosphäre laboratory as described in ref. 19. $^{11}CO_2$ was applied to the third fully developed source leaf, where it was rapidly incorporated in sucrose, the major form of C transport in the phloem. Plants were shielded with lead and tungsten to separately measure shoot and root activity with scintillation counters before and after treatments. For further details, see *Supporting Text*.

Enzyme Activity and Sugar Measurements. For measurements of soluble sugars (sucrose, glucose, and fructose) and enzyme activities [SuSy, soluble acid (vacuolar) invertase, cell wall invertase, and soluble alkaline (cytosolic) invertase], tissue samples were frozen in liquid nitrogen and homogenized. Sugars were measured after ethanol extraction according to ref. 44. Activity levels of SuSy and invertases were measured in desalted extracts according to refs. 45 and 46, respectively.

We thank M. Lim for plant transformation, R. Bahulikar for TaqMan assays, J. Schumacher for help with statistics, A. Steppuhn for comments, D. Schwachtje for help with Fig. 1, U. Schurr for financing the work of P.E.H.M. and S.J., and P. Geigenberger for financing the work of J.T.v.D. This work was supported by the Max-Planck-Gesellschaft.

1. Karban, R. & Baldwin, I. T. (1997) *Induced Responses to Herbivory* (Univ. of Chicago Press, Chicago).
2. Tiffin, P. (2000) *Evol. Ecol.* **14,** 523–536.
3. Stowe, K. A., Marquis, R. J., Hochwender, C. G. & Simms, E. L. (2000) *Annu. Rev. Ecol. Syst.* **31,** 565–595.
4. Strauss, S. Y., Watson, W. & Allen, M. T. (2003) *Ecology* **84,** 2074–2082.
5. Mauricio, R. (2000) *Evol. Ecol.* **14,** 491–507.
6. Tiffin, P. & Rausher, M. D. (1999) *Am. Nat.* **154,** 700–716.
7. Rausher, M. D. (2001) *Nature* **411,** 857–864.
8. Strauss, S. Y. & Agrawal, A. A. (1999) *Trends Ecol. Evol.* **14,** 179–185.
9. Weinig, C., Stinchcombe, J. R. & Schmitt, J. (2003) *Evolution (Lawrence, Kans.)* **57,** 1270–1280.
10. Trumble, J. T., Kolodnyhirsch, D. M. & Ting, I. P. (1993) *Annu. Rev. Entomol.* **38,** 93–119.
11. Baldwin, I. T. (2001) *Plant Physiol.* **127,** 1449–1458.
12. Morris, C. E. (1983) *J. Insect Physiol.* **29,** 807–817.

13. Halitschke, R. & Baldwin, I. T. (2003) *Plant J.* **36,** 794–807.
14. Halitschke, R., Gase, K., Hui, D., Schmidt, D. D. & Baldwin, I. T. (2003) *Plant Physiol.* **131,** 1894–1902.
15. Kessler, A., Halitschke, R. & Baldwin. I. T. (2004) *Science* **305,** 665–668.
16. van Dam, N. M., Hermenau, U. & Baldwin, I. T. (2001) *Ecol. Entomol.* **26,** 578–586.
17. Kessler, A. & Baldwin, I. T. (2002) *Ecology* **83,** 2346–2354.
18. Halitschke, R., Kessler, A., Kahl, J., Lorenz, A. & Baldwin, I. T. (2000) *Oecologia* **124,** 408–417.
19. Minchin, P. E. H. & Thorpe, M. R. (2003) *Funct. Plant Biol.* **30,** 831–841.
20. McCloud, E. S. & Baldwin, I. T. (1997) *Planta* **203,** 430–435.
21. Roda, A., Halitschke, R., Steppuhn, A. & Baldwin, I. T. (2004) *Mol. Ecol.* **13,** 2421–2433.
22. Halitschke, R., Schittko, U., Pohnert, G., Boland, W. & Baldwin, I. T. (2001) *Plant Physiol.* **125,** 711–717.
23. Roitsch, T. (1999) *Curr. Opin. Plant Biol.* **2,** 198–206.
24. Van Bel, A. J. E. (2003) *Plant Cell Environ.* **26,** 125–149.
25. Gould, N., Thorpe, M. R., Koroleva, O. & Minchin, P. E. H. (2005) *Funct. Plant Biol.* **32,** 1019–1026.
26. Sergeeva, L. I., Keurentjes, J. J. B., Bentsink, L., Vonk, J., van der Plas, L. H. W., Koornneef, M. & Vreugdenhil, D. (2006) *Proc. Natl. Acad. Sci. USA* **103,** 2994–2999.
27. Hermsmeier, D., Schittko, U. & Baldwin, I. T. (2001) *Plant Physiol.* **125,** 683–700.
28. Halford, N. G., Hey, S., Jhurreea, D., Laurie, S., McKibbin, R. S., Zhang, Y. & Matthew, J. P. (2004) *J. Exp. Bot.* **55,** 35–42.
29. Baldwin, I. T. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 8113–8118.
30. Hardie, D. G., Carling, D. & Carlson, M. (1998) *Annu. Rev. Biochem.* **67,** 821–855.
31. Laurie, S. & Halford, N. G. (2001) *Plant Growth. Regul.* **34,** 253–265.
32. Shaw, R. J., Lamia, K. A., Vasquez, D., Koo, S. H., Bardeesy, N., DePinho, R. A., Montminy, M. & Cantley, L. C. (2005) *Science* **310,** 1642–1646.
33. Vincent, O., Townley, R., Kuchin, S. & Carlson, M. (2001) *Genes Dev.* **15,** 1104–1114.
34. Lovas, A., Bimbo, A., Szabo, L. & Banfalvi, Z. (2003) *Plant J.* **33,** 139–147.
35. Zavala, J. A., Patankar, A. G., Gase, K. & Baldwin, I. T. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 1607–1612.
36. Glawe, G. A., Zavala, J. A., Kessler, A., Van Dam, N. M. & Baldwin, I. T. (2003) *Ecology* **84,** 79–90.
37. Winz, R. A. & Baldwin, I. T. (2001) *Plant Physiol.* **125,** 2189–2202.
38. Kahl, J., Siemens, D. H., Aerts, R. J., Gäbler, R., Kühnemann, R., Preston, C. A. & Baldwin, I. T. (2000) *Planta* **210,** 336–342.
39. Kessler, A. & Baldwin, I. T. (2004) *Plant J.* **38,** 639–649.
40. Voelckel, C., Schittko, U. & Baldwin, I. T. (2001) *Oecologia* **127,** 274–280.
41. Voelckel, C. & Baldwin, I. T. (2004) *Plant J.* **38,** 650–663.
42. Weinig, C., Stinchcombe, J. R. & Schmitt, J. (2003) *Mol. Ecol.* **12,** 1153–1163.
43. Simms, E. L. (2000) *Evol. Ecol.* **14,** 563–570.
44. Geigenberger, P., Hajirezaei, M., Geiger, M., Deiting, U., Sonnewald, U. & Stitt, M. (1998) *Planta* **205,** 428–437.
45. Dorion, S., Lalonde, S. & Saini, H. S. (1996) *Plant Physiol.* **111,** 137–145.
46. Oliver, S. N., Van Dongen, J. T., Alfred, S. C., Mamun, E. A., Zhao, X. C., Saini, H. S., Fernandes, S. F., Blanchard, C. L., Sutton, B. G., Geigenberger, P., *et al.* (2005) *Plant Cell Environ.* **28,** 1534–1551.



Welcome Jens Schwachtje Home Feedback Support Log off First vi

FACULTY OF 1000 BIOLOGY
MAJOR ADVANCES. EXPERT OPINIONS.

My F1000 Biology | Browse the Faculties | Top 10s | Advanced Search | My Details | About | Faculty Member List

Must Read
F1000 Factor 4.8
EndNote
Download citation
Send page by email

SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots.
Schwachtje J, Minchin PE, Jahnke S, van Dongen JT, Schittko U, Baldwin IT
Proc Natl Acad Sci U S A 2006 Aug 22 **103**(34):12935-40 [abstract on PubMed] [citations on Google Scholar] [related articles] [full text] [order article]

Selected by | Steven Huber / Anne Osbourn
First evaluation 12 Sep 2006 | Latest evaluation 13 Sep 2006
Relevant Sections

Faculty Comments

Faculty Member	Comments
Steven Huber University of Illinois at Urbana-Champaign, United States of America PLANT BIOLOGY Hypothesis	**This interesting paper reports that simulated herbivory of Nicotiana attenuata results in increased transport of photosynthate to roots in a complex response that appears to involve down regulation of the beta-subunit of the SNF1-related protein kinase, GAL83.** Fatty acid-amino acid conjugates from larval oral secretions are shown to be the signal that regulates GAL83 in source leaves and, in some manner, also increases acid invertase activity in roots. While many important details remain to be elucidated, the working model provides some molecular insights as to how plants divert resources to better tolerate herbivore damage. **Competing interests:** None declared Evaluated 13 Sep 2006 How to cite this evaluation
Anne Osbourn John Innes Centre, United Kingdom PLANT BIOLOGY Hypothesis New Finding	**This paper makes an important link between primary metabolism and biotic stress tolerance in plants.** The authors used a short-lived carbon isotope to follow changes in sink-source relations in Nicotiana attenuata in response to simulated herbivore attack. Herbivore attack increased the allocation of sugars to the roots, so generating enhanced root reserves that have the potential to support tolerance. This response is regulated by an SNF1-related kinase, a class of enzymes that is known to be important for regulation of sugar metabolism in diverse organisms. **Competing interests:** None declared Evaluated 12 Sep 2006 How to cite this evaluation

Faculty Comments

Manuscript II

Reverse Genetics in Ecological Research

Jens Schwachtje[*], Susan Kutschbach[*], Ian T. Baldwin[*†]

Accepted by PloS ONE

[*]*Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, 07745 Jena, Germany*

[†]*To whom correspondence should be addressed. E-mail: baldwin@ice.mpg.de*

Keywords: *Nicotiana attenuata,* reverse genetics, transformation, ecological research, empty vector controls, trypsin proteinase inhibitors.

Summary

By precisely manipulating the expression of individual genetic elements thought to be important for ecological performance, reverse genetics has the potential to revolutionize plant ecology. However, untested concerns about possible side-effects of the transformation technique, caused by *Agrobacterium* infection and tissue culture, on plant performance have stymied research by requiring onerous sample sizes. We compare 5 independently transformed *Nicotiana attenuata* lines harboring empty vector control (EVC) T-DNA lacking silencing information with isogenic wild types (WT), and measured a battery of ecologically relevant traits, known to be important in plant-herbivore interactions: phytohormones, secondary metabolites, growth and fitness parameters under stringent competitive conditions, and transcriptional regulation with microarrays. As a positive control, we included a line silenced in trypsin proteinase inhibitor gene (TPI) expression, a potent anti-herbivore defense known to exact fitness costs in its expression, in the analysis. The experiment was conducted twice, with 10 and 20 biological replicates per genotype. For all parameters, we detected no difference between any EVC and WT lines, but could readily detect a fitness benefit of silencing TPI production. A statistical power analyses revealed that the minimum sample sizes required for detecting significant fitness differences between EVC and WT was 2-3 orders of magnitude larger than the 10 replicates required to detect a fitness effect of TPI silencing. We conclude that possible side-effects of transformation are far too low to obfuscate the study of ecologically relevant phenotypes.

Introduction

Reverse genetics, the creation of a phenotype by gene silencing, has rapidly become the method of choice among physiologists for understanding the *in vivo* function of genes. Recent and dramatic advances in our understanding of the cellular function of small RNAs (siRNAs, miRNAs, etc.) in regulating gene expression have allowed for the development of transformation constructs (RNAi, inverted-repeat, antisense, artificial miRNA, etc.), which silence genes with great precision. With the appropriate choice of sequence, constructs can specifically silence individual genes in large gene families as long as a unique 22-24bp sequence can be identified. At the other extreme, all of the members of a gene family can be silenced with constructs harboring 22-24bp stretches of sequence shared by all family members [1,2]. These constructs are easily designed and can be stably or transiently introduced into the genomes of any organism for which transformation systems are available.

In contrast to their rapid adoption by physiologists, these reverse genetic tools have been adopted more slowly by ecologists and evolutionary biologists in their work on the whole-organismic and ecological consequences of gene function. The reasons are likely two-fold: first, a majority of researchers believe that in the evolution of the phenotype, quantitative trail loci (QTLs) are more important than known protein-coding loci and consequently have relied on quantitative genetic techniques to identify QTLs. Second, potential genomic side-effects caused by the transformation process are widely believed to obfuscate the functional analysis of genes that are responsible for specific traits [3]. Dramatic advances in our understanding of the molecular control of complex multi-genic traits have rapidly made the first concern a non-issue. The second, however, remains, and questions regarding how best to control for potential side-effects are relevant.

It is relatively easy to identify genomic effects that result from transformation procedures; many examples have been reported with the widely used *Agrobacterium* transformation system used to infect plant tissue with disarmed *A. tumefaciens* bacteria that integrate modified vector plasmids into the plant's nuclear genome [4,5]. The transferred DNA (T-DNA) is modified to include the sequence required for silencing the endogenous gene and a selective marker, most commonly an antibiotic resistance gene. *A. tumefaciens* appears to integrate T-DNA into random sites in the nuclear genome, and the insertion process may alter chromosome architecture or DNA sequence. Loss of gene function could result from the insertion of T-DNA into functional gene sequences, or the T-DNA may have pleiotropic effects on the expression of other genes [6,7]. In addition, genomic changes can

result from the tissue culture procedures that are required to transform several plant species, leading to epigenetic and heritable (somaclonal) variation of nucleic DNA [8-10]. Clearly, transformation-related alterations of DNA occur to different degrees [7]; yet surprisingly few studies have examined their consequences for whole-plant traits, such as fitness and ecological performance (measured by growth and reproduction, defense and tolerance, and competitive ability). If these reverse genetic procedures are to be used in ecological research, knowing how to most efficiently determine whether these unintended side-effects of transformation confound the analysis of the effects of the targeted gene will be crucial. The question is a quantitative one that requires balancing the number of independently transformed lines against the magnitude of the expected fitness effects. When genes are studied that result in fitness effects sufficiently large to be easily quantified over short time scales, e.g. one growing season, and in experiments with low numbers of replicates, minor molecular changes resulting from the transformation procedure are unlikely to be influential, if the necessary precautions have been followed with transformation procedure.

Several controls and precautions are commonly used to reduce the risk of confounding the unintended effects of transformation with the effects of silencing the expression of a given gene. Insertional mutations can be minimized when plants carry only a single T-DNA insertion. Some vector constructs allow the location of the T-DNA in the nuclear genome to be identified and determine whether a particular gene has been disrupted. An effective but more laborious strategy to rule out unintended effects is to use several independently transformed lines. If T-DNA insertion occurs at random places in the genome, the chances of disrupting the same gene or even different genes which confound the expected RNAi-mediated phenotype in a similar fashion in two independent transformations is extremely small. Backcrossing transgenic plants to their wild-type (WT) parents will also minimize possible transformation effects. However, not all of the variance that results from the transformation procedure is unhelpful for ecological analyses. For example, the variation in transgene expression resulting from inserting the T-DNA into different parts of the genome which have different levels of transcriptional activity (the so-called “positional effects”) can result in lines in which genes are silenced with different degrees of efficiency, despite being transformed with the same T-DNA construct [11]. This variation can be particularly useful for ecological studies: the accumulation of transcripts of the targeted gene can be quantified in several independently transformed lines, not only to demonstrate that the targeted gene was in fact silenced but also to examine the quantitative relationship between gene expression and phenotype. Moreover, including plants transformed with an empty vector construct as

controls -- plants which have undergone the transformation procedure and carry a T-DNA that lacks the information for silencing of a specific gene but contains all other information necessary for gene silencing, including the antibiotic resistance gene -- is thought to be essential for the analysis of transformed plants.

Here we examine the general issue of how many empty vector controls (EVCs) are necessary to estimate the potential unintended effects of the transformation process for ecologically relevant traits. We ask whether the procedure used in our laboratory to transform *Nicotiana attenuata*, a native annual from North America, results in unintended effects on a suite of ecologically relevant herbivore-resistance traits, plant growth and reproductive performance traits. We use plants that have experienced tissue culture and *A. tumefaciens* infection, and that carry a single insertion of an empty vector T-DNA, including a hygromycin-resistance gene. Five independently transformed homozygous EVC lines are compared with isogenic wild types of the same generation of *N. attenuata* in a competition experimental design optimized to identify subtle differences in growth and fitness. The experimental set-up, which involves competing two size-matched seedlings in a single 2 L pot, was designed to simulate the competition for soil nutrients and water that occurs for *N. attenuata* as it germinates synchronously from long-lived seed banks after fires, such as characterize its natural niche in the Great Basin Desert of North America [12]. The competition experiments were carried out two times, with 20 and 10 replicates. The application of methyl jasmonate (MJ), a standardized and reproducible treatment, is known to elicit herbivore defense responses [13-16]. We quantified traits that provide demonstrably useful proxies for plant fitness in competitive (height and seed capsule production) and herbivore-intensive environments [phytohormones (JA, JA-Ile) responsible for eliciting the plant's direct defenses, nicotine, and trypsin proteinase inhibitor (TPI) activity], and we hybridized microarrays for a large-scale analysis of potential transcriptional effects. To determine the number of replicates required to detect significant changes in fitness parameters resulting from silencing a defense gene, we included as a positive control transgenic plants that had undergone the same transformation procedure as the EVCs but carried a single insertion of an inverted-repeat construct to silence a TPI gene by RNAi. In previous glasshouse studies, when this gene was silenced in a native *N. attenuata* ecotype that produced TPIs, seed capsule production significantly increased, and when TPI expression was restored by transforming an ecotype that was naturally deficient in TPI expression, seed capsule production decreased [17].

Results

Reproductive performance and growth

Results from previous studies with *N. attenuata* plants silenced and ectopically expressing TPIs [17] revealed that in competition experiments, 10 replicates are sufficient to reveal significant differences in lifetime seed capsule production among TPI-expressing and TPI-silenced pairs. No significant differences were found between WT and EVC pairs in the previous analyses with paired t-tests. Here we increased our statistical power to detect fitness-related differences among WT and EVC pairs by using 20 replicates. Each of the 5 EVC lines, the irPI line and WT were paired with WT in a 2 L pot after being size matched. The paired design was chosen to impose competition on plant pairs: the limited resources of a 2L pot strongly amplify small differences in growth rates, resource allocation, or competitive ability between the initially same-sized plants [18]. For the three genotype pairings that were elicited with MJ (see Fig. 1), we elicited the plants twice to increase defense responses and their associated costs, to increase the chance of detecting spurious plant growth and performance effects in the competition set-up. To compare differences between plant pairings (two sample t-test), for each plant pair the two values of a measured trait were subtracted from each other, and the mean difference for all replicates of a pairing is referred to as Δ, combined with subscripts that describe the measured trait (e. g. $\Delta_{nicotine}$). For paired t-tests we compared means of the measured trait of each of the two lines of a pairing.

As expected, we found $\Delta_{seed\ capsules}$ of the irPI-WT pairing to be significantly larger compared to $\Delta_{seed\ capsules}$ of the WT-WT pairing after MJ induction (ANOVA: $F_{9,190} = 5.825$, $P < 0.0001$; post-hoc test: Tukey/Kramer: $P < 0.0001$, Fig. 1A), because irPI plants produced more seed capsules than did WT and EVC plants. Clearly, silencing this potent defense significantly increases *N. attenuata*'s reproductive performance, a result consistent with a significant cost of TPI production.

No other pairings showed significant differences of $\Delta_{seed\ capsules}$ compared to WT-WT pairings. (ANOVA: $F_{8,171} = 0.330$, $P = 0.9537$). Moreover, no differences in Δ_{height} could be detected (ANOVA: $F_{9,190} = 0.863$, $P = 0.5594$, Fig. 1B). These results demonstrate that none of the 5 EVC differed from WT plants in any growth-related measure in a competition design which is designed to detect smaller differences in performance than those that can be detected when plants are grown in single pots [19].

Paired t-tests for seed capsules of MJ treated plants resulted in $t = -0.223$; $P = 0.8257$; DF = 19 for EVC - WT pairings, and $t = 5.276$; $P < 0.0001$; DF = 19 for irPI - WT pairings. In a second competition experiment, replicated 10 times and with the same pairings as the first experiment, plants were grown during the short-day season of the year; this schedule resulted in lower absolute amounts of seed capsules per plant (20 % fewer; 39.3 vs. 47.2 seed capsules on average) than in the experiment with 20 replicates. Paired t-tests for this experiment for seed capsules of MJ treated plants resulted in $t = -0.072$; $P = 0.9444$; DF = 9 for EVC - WT pairings and $t = 2.613$; $P = 0.0281$; DF = 9 for irPI - WT pairings.

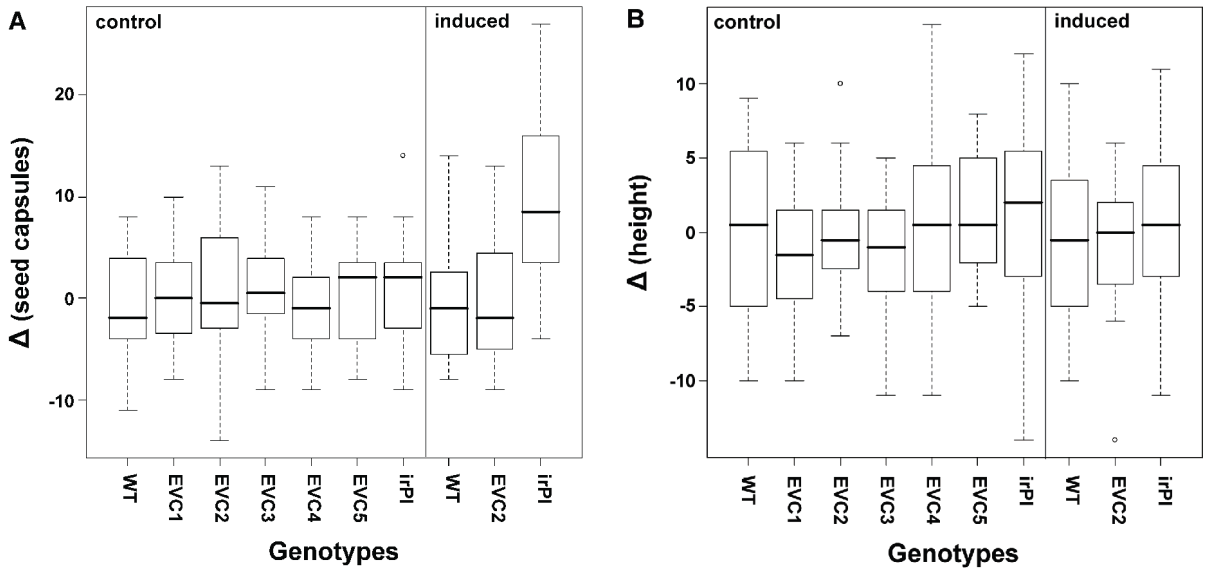


Fig. 1: A, B: Box-plots of differences (Δ) in (**A**) lifetime seed capsules (number) and (**B**) height (cm), among pairs of initially size-matched plants competing in a 2-L pot (control: untreated; induced: MJ-treated). Every isogenic plant of a genotype noted on the x-axis was paired with an isogenic WT plant. N = 20; for statistical evaluation see text. Whiskers designate the 95 % confidence interval of data.

Power analyses

To determine the minimum sample sizes necessary to detect significant differences between $\Delta_{\text{seed capsules}}$ of irPI - WT and WT - WT, and between $\Delta_{\text{seed capsules}}$ of EVC - WT and WT - WT (two sample t-test), power analyses (for $\beta \leq 0.2$) were carried out with the data from the two experiments (MJ-treated plants). In addition, power analyses (for $\beta \leq 0.2$) were carried out based on paired t-tests (mean difference of one pairing), as this is the statistical test we use to analyze performance effects of transformed plants in glasshouse competition experiments as well as in field trials [17,20].

Calculated sample sizes necessary to detect differences between $\Delta_{\text{seed capsules}}$ of EVC - WT and WT - WT, based on the experiment with 20 replicates, resulted in N = 8865 (1-β = 0.8, Fig. 2), and calculated sample sizes necessary to detect significant differences between $\Delta_{\text{seed capsules}}$ of irPI - WT and WT - WT resulted in N = 9 (1-β = 0.82, Fig. 2). Based on the two means of a paired t-test, least sample size for the irPI - WT pairing resulted in N = 7 (1-β = 0.866), for the EVC - WT pairing in N = 2186 (1-β = 0.8), and in N = 2536 (1-β = 0.8) for the WT - WT pairing.

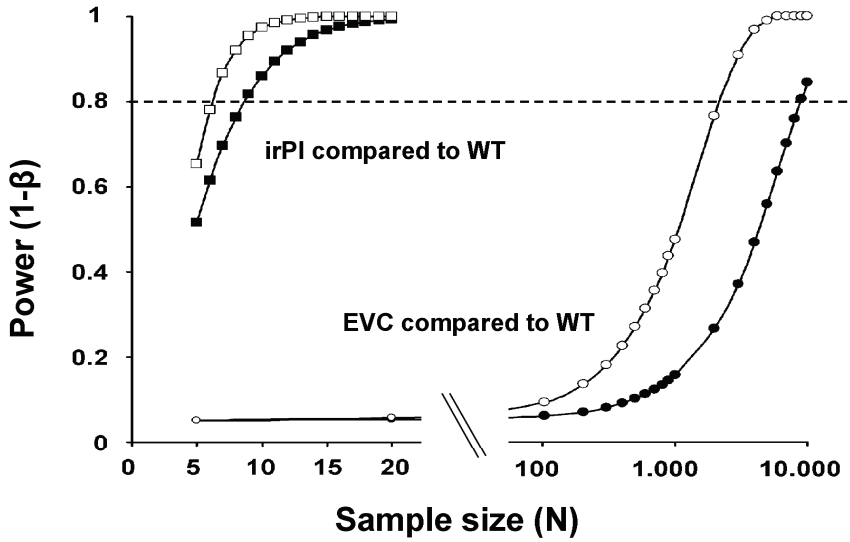


Fig. 2: Calculated statistical power (dotted line: power = 0.8) as a function of sample size for detection of significant differences for $\Delta_{\text{seed capsules}}$ (two sample t-test) between irPI - WT and WT - WT (closed squares), and EVC - WT and WT - WT (closed circles), and for significant differences between two means (paired t-test) of irPI - WT (open squares) and EVC - WT (open circles), based on seed capsule data from the first experiment (N = 20). Note that the scale of the x-axis is diverted in a linear (left) and a logarithmic (right) segment.

For the second competition experiment with 10 replicates, sample size calculation for differences between $\Delta_{\text{seed capsules}}$ of EVC - WT and WT - WT resulted in N = 2602 (1-β = 0.8) and for differences between $\Delta_{\text{seed capsules}}$ of irPI - WT and WT - WT in N = 17 (1-β = 0.81). Based on a paired t-test least sample sizes for the EVC - WT pairing were N = 8718 (1-β = 0.8), and for the irPI - WT pairing N = 13 (1-β = 0.828).

Nicotine levels and TPI activity

Nicotine levels and TPI activity were measured in the 10 replicate experiment. A Levene-test revealed no homoscedasticity of Δ_{nicotine} and $\Delta_{\text{TPI activity}}$ for control and induced

pairings; thus, these two groups were analyzed separately. Levene-tests for control (uninduced) pairings were non-significant for $\Delta_{nicotine}$ ($F_{6,63} = 1.404$, $P = 0.238$) and $\Delta_{TPI\ activity}$

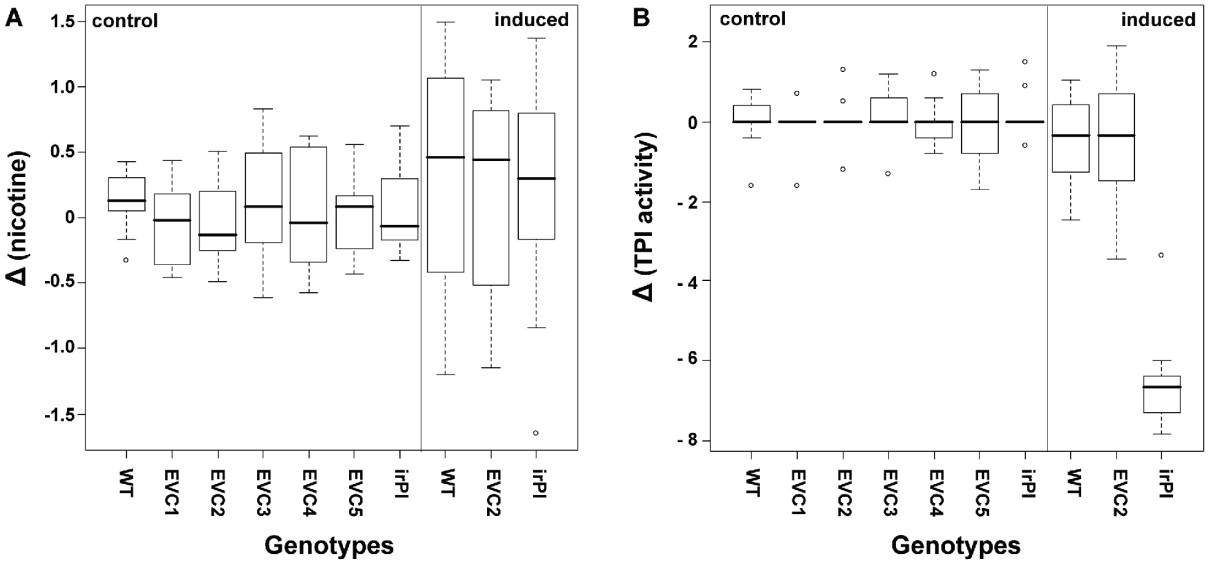


Fig. 3 A, B: Box-plots of differences (Δ) in (**A**) nicotine content (mg/g fresh mass) and (**B**) PI activity (nmol/mg protein) among pairs of initially size-matched plants competing in a 2-L pot (control: untreated; induced: MJ-treated). Every isogenic plant of a genotype noted on the x-axis was paired with an isogenic WT plant. N = 10; for statistical evaluation see text. Whiskers designate the 95 % confidence interval of data.

($F_{6,63} = 0.729$, $P = 0.607$). We did not find significant differences between the $\Delta_{nicotine}$ of uninduced plants in any pairing (ANOVA: $F_{6,63} = 0.352$; $P = 0.954$, Fig. 3A), and $\Delta_{TPI\ activity}$ (ANOVA: $F_{6,63} = 0.179$, $P = 0.982$, Fig. 3B). MJ induction did not influence the homoscedasticity of $\Delta_{nicotine}$ and the $\Delta_{TPI\ activity}$ of plant pairs (Levene-test: nicotine: $F_{2,27} = 0.180$, $P = 0.838$; TPI activity: $F_{2,27} = 1.169$, $P = 0.326$). $\Delta_{nicotine}$ was not different between induced pairings (ANOVA: $F_{2,27} = 0.046$, $P = 0.956$, Fig. 3A), but $\Delta_{TPI\ activity}$ was significantly different (ANOVA: $F_{2,27} = 70.19$, $P < 0.0001$; post-hoc test: Tukey/Kramer for irPI-WT and WT-WT pairings: $p < 0.0001$, for EVC2-WT and WT-WT pairings: $P = 0.997$, Fig. 3B). As expected, the TPI activity of MJ-induced irPI plants was not detectable [21], demonstrating that the TPI gene had been effectively silenced by the transformation with the inverted-repeat construct.

Microarrays

We hybridized 21 microarrays, consisting of three biological replicates, for all 7 genotypes, to determine if transcript accumulation 24 h after treatment with MJ differed

among the genotypes. An ANOVA (2000 permutations, alpha: 0.01) revealed 16 significantly differently regulated genes (including 7 PI genes), most of them in the irPI microarrays (Fig. 4A, for a list of genes, see Supplemental Information). Most genes (1385) were not differentially regulated in any plant line.

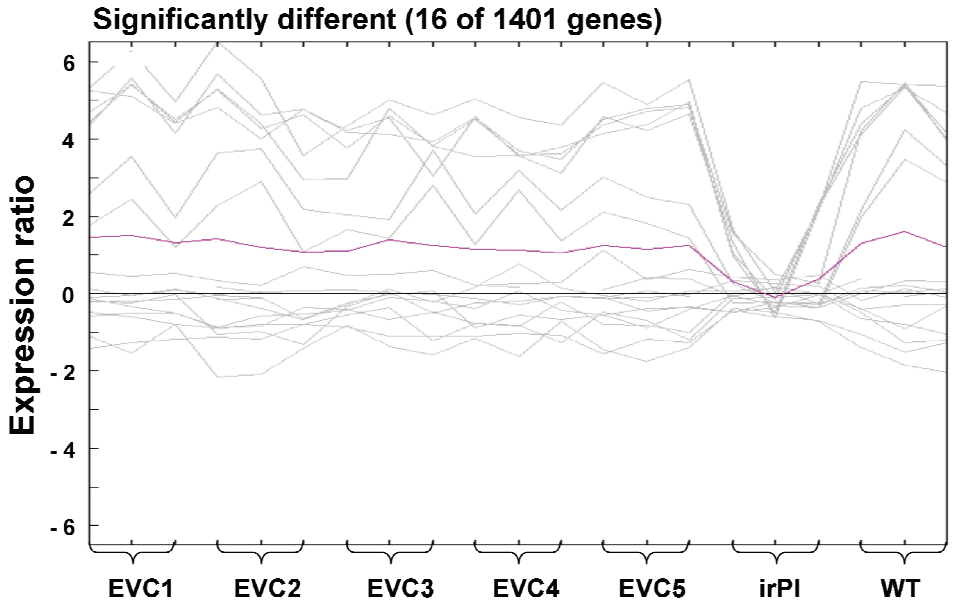


Fig. 4: Graph of expression ratios (log_2 transformed) of significantly different expressed genes in source leaves of each genotype 24 h after elicitation with MJ. Microarrays were hybridized with three different biological replicates for each genotype (the MJ-treated sample was labeled with cy3, controls with cy5). ANOVA, 2000 permutations, α: 0.01. For a list of different and non-different genes, see Supplemental Information.

Hormones

JA and its amino acid conjugates (JA-Ile) play crucial roles in the defense signaling of plants [22], regulating nicotine and TPI production, as well as a variety of other defense traits, such as volatile emission [19]. When plants were elicited by wounding and immediately applying caterpillar regurgitant to the wounds, the elicitation of JA and JA-Ile/Leu conjugates did not differ among all genotypes (JA: ANOVA; $F_{6,14}$: 0.664; $P = 0.681$; JA-Ile/Leu: ANOVA; $F_{6,14}$: 0.997; $P = 0.465$, see Supplemental Fig. 1).

Discussion

The objective of this study was to evaluate the effect of transformation, based on tissue culturing and *A. tumefaciens* infection, for possible side-effects on ecologically relevant performance traits, in order to determine how many and which controls are needed in

functional ecological experiments with gene-silenced *N. attenuata* plants. This issue is of particular importance for ecologists, because if the power of reverse genetics is to be realized for field studies, where large sample sizes can be onerous, it is important to determine the effort that is required to evaluate the possibility that side-effects of the transformation process confound the performance analysis of a gene of interest.

We examined the performance of 5 independently transformed EVC lines in comparison to their isogenic WT counterparts of *N. attenuata*, with a variety of ecologically relevant traits including growth, reproductive output, defense metabolites and MJ-elicited transcriptional responses of numerous secondary and primary genes with microarrays. In order to increase our ability to detect subtle differences, a competition set-up was used which paired transformed plants with isogenic WT plants. As a positive control for gene silencing effects, we included pairings with an irPI line that lacks a TPI activity and is known to have significantly increased reproductive performance [17]. In no experiments were significant differences between EVC and WT plants detectable for any measured traits: hormone levels, nicotine levels (Fig. 3A), TPI activity (Fig. 3B), transcriptional profiles of a large number of genes (Fig. 4), height and reproductive (Darwinian) fitness (Fig. 1 A, B). Darwinian fitness, the crucial parameter in functional ecological studies, was estimated by the number of seed capsules produced. This number expresses a plant's fitness through the female function, which gives an accurate fitness estimate especially for this largely self-pollinating species [23], and reflects the total of all effects, positive and negative, on a plant's reproductive capacity during growth under intense competition with an isogenic WT plant. Subtle differences in plant growth are frequently amplified when plants are grown in competition; note for example, the fitness costs associated with MJ elicitation [18]. We conclude that regardless of the unmeasured molecular side-effects that might have occurred during tissue culturing and *Agrobacterium* transformation of EVC lines, none significantly influenced any of these ecologically important traits.

A power analysis (for $\beta \leq 0.2$) of the number of replicates required to detect fitness differences between EVC or irPI and WT lines based on the 20-replicate experiment revealed just how small the chances were of having transformation-related side-effects confound the analysis of TPI expression on reproductive performance. In order to detect differences between EVCs and WT plants, a sample size 3 orders of magnitude larger than that required to detect the effects of TPI-silencing would be necessary (Fig. 2). Based on a paired t-test, differences between competing WT plants could be detected with $N = 2536$ ($1-\beta = 0.8$) and

between competing WT and EVC plants with N = 2186 (1-β = 0.8), what clearly demonstrates how similar EVC and WT plants are.

The difference of required sample sizes was less pronounced when the power analysis was conducted with the data from the 10-replicate experiment conducted during the short-day period of the year, where plants produced 20 % fewer capsules; however, the TPI effect is still dramatically larger than the differences between EVC and WT.

How many independent EVC lines should be included to test for transformation effects, and how many replicates are necessary to detect significant ecological effects of single genes? To answer this question, the work of Tian *et al.* [24] is often cited as the standard. This study used an elegant Cre/Lox transformation system for *Arabidopsis* with backcrossed controls, a variety of controls that examine the effects of T-DNA insertions, and 500 replicates to measure the large fitness effect of an resistance (R) gene (9 % more seeds in plants lacking the R gene). While this is an exemplary study of the use of transgenic plants to study an ecological question, in our opinion, the study is over-controlled, and inappropriate to use as the standard to which all other studies should be held. The fitness cost of the R-gene could have been detected with many fewer replicates had the research been done as a traditional reverse-genetics study targeting the fitness consequences of a known gene. The sample size requirements of a QTL analysis to detect very small effect sizes are often confused with those required for a reverse-genetics study. Studies which aim to determine QTLs that influence adaptive ecological traits in the long-term context of evolution generally require a large number of replicates for statistically significant effects. Reverse genetics in ecology, however, aims to understand the effect on Darwinian fitness of an established trait, mostly related to one or few genes, and therefore will most often lead to much larger effects, which are comparatively easy to detect with lower numbers of replicates, as our power analysis demonstrated.

How many EVC lines should then be used for experiments in functional ecology? The fact that the differences of EVC and WT plants we describe here are extremely low compared to the great effects of silencing of the TPI gene (Fig. 2) suggests that the use of WT plants as controls is sufficient for ecological experiments with *N. attenuata* and that EVCs can be omitted. Time-consuming procedures, such as repeated backcrossing with WT parents to obtain “clean” transgenes, are not justified by the results of this study. The evaluation of several independently transformed lines is part of tests for side-effects of the transformation procedure. If the same quantifiable phenotype appears at least twice in different lines transformed with the same construct, the probability is extremely low that the phenotype will

be a result of unintended side-effects of transformation, especially, when the phenotype can be predicted from the biochemical function of the silenced gene.

Side-effects are often found in purely molecular analysis of transformed organisms, however, only few studies describe their translation to the whole-plant level. Generally, most effects fall in the natural range of variation [25]. One of the most carefully designed studies was done by Purrington and Bergelson [26], who used several independently transformed and double backcrossed EVCs carrying an antibiotic resistance gene. They could show that the seed production of transformed *Arabidopsis* plants engineered for antibiotic resistance did not differ from that of WT controls, and that the expression of the resistance gene is not associated with metabolic costs. This is an important finding, since antibiotic resistance genes are commonly used in reverse genetics. Ruebelt *et al*.[27] found that the differences of 2D seed protein profiles of *Arabidopsis* between wild types and several transformed plants were small and fell in the range of the differences among 12 Arabidopsis ecotypes. Rogan *et al*. [28] examined two types of transgenic virus-resistant potatoes and found them substantially equivalent to wild types within a variety of metabolites, nutrients and general morphological parameters. Another recent study compared various potato lines from two varieties and included WT plants, untransformed plants that have undergone tissue culture, EVCs, and plants with genes in both sense and antisense orientation [29]. Measurements were made of numerous primary and secondary metabolites, as well as of general parameters, such as dry mass and tuber numbers. Some significant but randomly distributed differences were reported among transgenic plants, tissue-cultured plants, and wild types, but none of these could be attributable to a specific construct. The most obvious differences existed between the two potato varieties. However, unintended changes caused by tissue culture appear to be a problem for some species [25]. Sandoval *et al*. [30] observed that dwarf types of Cavendish banana had lower endogenous gibberellin levels, affecting banana plant height, and Ramulu *et al*. [31] described DNA variation in micropropagated potato. However, in our study we did not detect any differences which could be attributed to tissue culturing.

Glasshouse experiments aim to mimic natural growth conditions; however, despite supplemental illumination from HID Na-vapor lamps, the variation of sunlight intensity and day length during the year may influence plant growth. To detect seasonal differences, we compared fitness data from two experiments on plants grown during short- and long-day seasons. When days were shorter, the total number of seed capsules produced per plants was 20 % fewer than from plants grown during a long-day period. With a power analysis, we could demonstrate that gene silencing effects (irPI vs.WT) of both experiments largely

outweighed differences between EVC and WT, but that the smallest number of replicates required for significant differences were slightly higher for irPI - WT differences compared to the experiment carried out during the long day season. None of the other measurements, e.g. of nicotine levels and of TPI activity, differed from measurements made in field studies. This demonstrates that the glasshouse environment sufficiently mimics conditions found in the natural environment of *N. attenuata*.

Our study demonstrates that laborious testing of transformed plants does not reveal any significant change in various ecologically relevant plant traits, or in the transcription of numerous ecologically and physiologically relevant genes. Since we generally examine genes that have marked effects on defense and fitness traits, which are detectable with relatively low numbers of replicates, we are convinced that reverse genetics is a powerful and accurate tool for ecological studies and that its potential to obfuscate intended gene silencing is very low. That the genome of *Nicotiana* species is very large and has a relatively low gene density (for example the *Nicotiana tabacum* genome is 20 times larger than that of *Arabidopsis thaliana* [32]), may be a reason for our failure to detect side-effects of transformation, because gene disruption might be a rare event in a large background of non-coding DNA. However, the genome characteristics of *Arabidopsis* may lead to a higher probability for side-effects of transformation, requiring more rigid controls for this species.

Material & Methods

Plant transformation

We used 5 independently transformed EVC lines containing the pRESC2NC vector construct that lacks the silencing information and carries a hygromycin-resistance gene, as described in [17,33]. The plants were regenerated from tissue culture after being transformed by *A. tumefaciens* (strain LBA4404, Life Technologies-Gibco BRL) as described in [34]. T_2 generations were used for the experiments. Transgenic plants were tested for homozygosity (by segregation analysis), diploidy (by flow cytometry), and single insertion of T-DNA (by Southern blotting). For more details, see Supplemental Information.

To analyze the phenotypic effects of gene silencing, we used as a positive control an irPI line that had undergone the same transformation procedure and contained an inverted-repeat (ir) silencing sequence for an *N. attenuata* TPI gene as described in [21]. All transformed plants stem from a wild-type inbred line of the 14th generation, which was originally collected from a plant growing near Santa Clara, Utah. Transformed plants used for

experiments were the T_2 generation. As isogenic controls, we used wild-type (WT) plants from the 17th generation of the inbred line.

Plant growth and fitness measures

N. attenuata plants were grown in the glasshouse under conditions described in [34,35] with a 16 h light: 8 h dark period and artificial light (335 μmol*m^{-2} *s^{-1} at 1.5 m distance). For both experiments, plants were potted in 2 L pots containing soil, with two plants per pot. The plant pairings included each of the 5 EVC lines [A-04-08 (EVC1), A-04-09 (EVC2), A-04-104 (EVC3), A-04-110 (EVC4), A-04-117 (EVC5)] paired with WT, irPI (A-04-186) paired with WT, and WT paired with WT. Rosette diameters were measured after plants grew for 8 days in the glasshouse, and for each plant combination, the best-matched pairs were selected.

For fitness measurements in the first experiment, the 20 best-matched pairs (of an initial 28) were chosen from each combination of genotypes. Plants were allowed to elongate fully for a 35-day growing period. At the end of the growing period, watering was reduced over 10 days as described in [36], to simulate the water conditions of *N. attenuata* at the end of the growing season in its natural habitat. After an additional 12 days, when all capsules were fully ripened, height and fitness parameters were measured; the latter was estimated by lifetime seed capsule production. This experiment was carried out during April-June, a season where sunlight and day-length enhance the PAR supplied by supplemental Na-vapor HID lamps of the glasshouse (19,20). In a second competition experiment, conducted during October-December when the PAR provided by natural sunlight is lower, but under the same supplemental lighting as above, we measured nicotine, TPI activity (by sampling one elicited leaf from all plants) and fitness. For this experiment, the 10 best-matched pairs (of 16) were chosen.

Nicotine levels and TPI activity measurements

At the rosette stage, directly after size matching, 10 pairs of WT - WT, irPI - WT, and EVC2 - WT were treated with 150 μg MJ dissolved in 20 μl lanolin as described in [37]. MJ, which is known to elicit defense responses very similar to those elicited by insect herbivory, was applied to the bases of 2 source leaves growing at nodes +1 and +3 [13-16]. Three days after MJ treatment, samples were taken from a systemic (non-treated) source leaf of each plant of the 3 induced and 7 control pairings (all 5 EVC, irPI and WT, each paired with WT)

and measured for nicotine levels (with HPLC) and TPI activity (with radial diffusion assays) according to procedures described in [38] and [18].

.

Hormone measurements

For phytohormone measurements, 6 plants of each genotype were grown individually in 1 L pots and analyzed at the rosette stage of growth. To elicit a change in phytohormone response in a highly reproducible manner and thereby allow subtle changes in phytohormone kinetics to be detected (see [22], for example), one source leaf in half of the plants of each line was wounded with a pattern wheel and 20 µl 1:5 water-diluted oral secretions and regurgitate (OS) from the specialist lepidopteran herbivore *Manduca sexta*, a species that regularly accounts for major losses of leaf area in native populations of *N. attenuata*, was applied. This treatment elicits defense responses similar to those of feeding *M. sexta* [13]. Induced and control samples were harvested 45 min after treatment, when jasmonic acid (JA) levels reach their maximum, and measured by LC/MS according to [39].

Microarray analysis

For microarray samples as for phytohormone analysis, additional plant pairs were established so that the sampling did not confound the measures of plant performance. Two source leaves per plant were treated at 11 a.m. with 150 µg MJ as described above. Leaves were harvested after 24 h and RNA was extracted as described in [36]. For each of the seven plant lines, 3 microarrays were hybridized to compare transcriptional profiles of elicited and unelicited leaves of plants of the same line. RNA was processed for microarray analysis and microarrays were hybridized as described in [20,40,41]. RNA from MJ elicited leaves was labeled with cy3; RNA from the control leaves of unelicited plants of the same line was labeled with cy5. Approximately 400 µg total RNA was used in each labeling reaction. The microarray was enriched with *N. attenuata* genes, which are known to be responsive to *M. sexta* attack [14,35,40-43]. Microarray data were lowess-normalized with the MIDAS package [44]. The quadruplicate spots of each gene were analyzed for significant differences using a t-test at a confidence level (α) of 0.05, and a threshold of a 1.5-fold change in expression ratio was used. For statistical analysis, all 21 microarrays were analyzed with the TMEV software [44].

Statistical analysis

Samples were evaluated with a two sample t-test (and a paired t-test. Differences among traits of the paired set-up were analyzed by a Levene-test for homogeneity and subsequent analysis of variances (ANOVA). Unless otherwise noted, Levene tests revealed no significant heteroscedasticity. The Tukey/Kramer test was used as a post-hoc test. Statistics were carried out by using Statview (www.jmp.com) and the free statistics software, R (www.r-project.org). For power analyses, we used the statistical software PASS (www.ncss.com).

Acknowledgements

We thank J. Schumacher for help with statistics, A. Steppuhn for comments, and S. Allmann for help with microarray processing. This work was supported by the Max-Planck-Society.

Literature

1. Miki D, Itoh R, Shimamoto K (2005) RNA silencing of single and multiple members in a gene family of rice. Plant Physiology 138: 1903-1913.
2. Watson JM, Fusaro AF, Wang MB, Waterhouse PM (2005) RNA silencing platforms in plants. Febs Letters 579: 5982-5987.
3. Cellini F, Chesson A, Colquhoun I, Constable A, Davies HV, et al. (2004) Unintended effects and their detection in genetically modified crops. Food and Chemical Toxicology 42: 1089-1125.
4. Gelvin SB (2003) *Agobacterium*-mediated plant transformation: The biology behind the "gene-Jockeying" tool. Microbiology and Molecular Biology Reviews 67: 16-37.
5. Tzfira T, Citovsky V (2006) Agrobacterium-mediated genetic transformation of plants: biology and biotechnology. Current Opinion in Biotechnology 17: 147-154.
6. Forsbach A, Schubert D, Lechtenberg B, Gils M, Schmidt R (2003) A comprehensive characterization of single-copy T-DNA insertions in the Arabidopsis thaliana genome. Plant Molecular Biology 52: 161-176.
7. Latham JR, Wilson AK, Steinbrecher RA (2006) The mutational consequences of plant transformation. Journal of Biomedicine and Biotechnology: Art. No. 25376 22006.
8. Bhat SR, Srinivasan S (2002) Molecular and genetic analyses of transgenic plants: Considerations and approaches. Plant Science 163: 673-681.
9. Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology 43: 179-188.
10. Larkin PJ, Scowcroft WR (1981) Somaclonal variation - a novel source of variability from cell-cultures for plant improvement. Theoretical and Applied Genetics 60: 197-214.
11. Butaye KMJ, Cammue BPA, Delaure SL, De Bolle MFC (2005) Approaches to minimize variation of transgene expression in plants. Molecular Breeding 16: 79-91.
12. Preston CA, Baldwin IT (1999) Positive and negative signals regulate germination in the post-fire annual, *Nicotiana attenuata*. Ecology 80: 481-494.
13. Halitschke R, Gase K, Hui DQ, Schmidt DD, Baldwin IT (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-

specific transcriptional changes are mediated by fatty acid-amino acid conjugates. Plant Physiology 131: 1894-1902.
14. Hui DQ, Iqbal J, Lehmann K, Gase K, Saluz HP, et al. (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*: V. Microarray analysis and further characterization of large-scale changes in herbivore-induced mRNAs. Plant Physiology 131: 1877-1893.
15. Roda AL, Baldwin IT (2003) Molecular technology reveals how the induced direct defenses of plants work. Basic and Applied Ecology 4: 15-26.
16. Schittko U, Hermsmeier D, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. II. Accumulation of plant mRNAs in response to insect-derived cues. Plant Physiology 125: 701-710.
17. Zavala JA, Patankar AG, Gase K, Baldwin IT (2004) Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata*. Proceedings of the National Academy of Sciences of the United States of America 101: 1607-1612.
18. Van Dam NM, Horn M, Mares M, Baldwin IT (2001) Ontogeny constrains systemic protease inhibitor response in *Nicotiana attenuata*. Journal of Chemical Ecology 27: 547-568.
19. Halitschke R, Ziegler J, Keinanen M, Baldwin IT (2004) Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling crosstalk in *Nicotiana attenuata*. Plant Journal 40: 35-46.
20. Pandey SP, Baldwin IT (2007) RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of *Nicotiana attenuata* to herbivore attack in nature. Plant Journal 50: 40-53.
21. Steppuhn A, Baldwin IT (2007) Resistance management in a native plant: Nicotine prevents herbivores from compensating for plant protease inhibitors. Ecology Letters 10: 499-511.
22. Kang JH, Wang L, Giri A, Baldwin IT (2006) Silencing threonine deaminase and JAR4 in *Nicotiana attenuata* impairs jasmonic acid-isoleucine-mediated defenses against *Manduca sexta*. Plant Cell 18: 3303-3320.
23. Sime KR, Baldwin, I. T. (2003) Opportunistic out-crossing in *Nicotiana attenuata*. BMC Ecology 3: Art. 6.
24. Tian D, Traw MB, Chen JQ, Kreitman M, Bergelson J (2003) Fitness costs of R-gene-mediated resistance in *Arabidopsis thaliana*. Nature 423: 74-77.
25. Filipecki M, Malepszy S (2006) Unintended consequences of plant transformation: a molecular insight. Journal of Applied Genetics 47: 277-286.
26. Purrington CB, Bergelson J (1997) Fitness consequences of genetically engineered herbicide and antibiotic resistance in *Arabidopsis thaliana*. Genetics 145: 807-814.
27. Ruebelt MC, Lipp M, Reynolds TL, Schmuke JJ, Astwood JD, et al. (2006) Application of two-dimensional gel electrophoresis to interrogate alterations in the proteome of gentically modified crops. 3. Assessing unintended effects. Journal of Agricultural and Food Chemistry 54: 2169-2177.
28. Rogan GJ, Bookout JT, Duncan DR, Fuchs RL, Lavrik PB, et al. (2000) Compositional analysis of tubers from insect and virus resistant potato plants. Journal of Agricultural and Food Chemistry 48: 5936-5945.
29. Shepherd LVT, McNicol JW, Razzo R, Taylor MA, Davies HV, et al. (2006) Assessing the potential for unintended effects in genetically modified potatoes perturbed in metabolic and developmental processes. Targeted analysis of key nutrients and anti-nutrients. Transgenic Research 15: 409-425.

30. Sandoval J, Kerbellec F, Cote F, Doumas P (1995) Distribution of endogenous gibberellins in dwarf and giant off-types banana (*Musa banana*, Cv Grand Nain) plants from *in vitro* propagation. Plant Growth Regulation 17: 219-224.
31. Ramulu KS, Dijkhuis P, Tencate CHH, Degroot B (1985) Patterns of DNA and chromosome variation during *in vitro* growth in various genotypes of potato. Plant Science 41: 69-78.
32. Kirik A, Salomon S, Puchta H (2000) Species-specific double-strandbreak repair and genome evolution in plants. EMBO Journal 19: 5562-5566.
33. Bubner B, Gase K, Berger B, Link D, Baldwin IT (2006) Occurrence of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue. Plant Cell Reports 25: 668-675.
34. Krugel T, Lim M, Gase K, Halitschke R, Baldwin IT (2002) Agrobacterium-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. Chemoecology 12: 177-183.
35. Hermsmeier D, Schittko U, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. Plant Physiology 125: 683-700.
36. Schwachtje J, Minchin PEH, Jahnke S, van Dongen JT, Schittko U, et al. (2006) SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. Proceedings of the National Academy of Sciences of the United States of America 103: 12935-12940.
37. Baldwin IT, Schmelz EA, Ohnmeiss TE (1994) Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* Spegazzini and Comes. Journal of Chemical Ecology 20: 2139-2157.
38. Keinanen M, Oldham NJ, Baldwin IT (2001) Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. Journal of Agricultural and Food Chemistry 49: 3553-3558.
39. Wu JQ, Hettenhausen C, Meldau S, Baldwin IT (2007) Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*. Plant Cell 19: 1096-1122.
40. Voelckel C, Baldwin IT (2004) Generalist and specialist lepidopteran larvae elicit different transcriptional responses in *Nicotiana attenuata*, which correlate with larval FAC profiles. Ecology Letters 7: 770-775.
41. Voelckel C, Baldwin IT (2004) Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations. Plant Journal 38: 650-663.
42. Heidel AJ, Baldwin IT (2004) Microarray analysis of salicylic acid- and jasmonic acid-signalling in responses of *Nicotiana attenuata* to attack by insects from multiple feeding guilds. Plant Cell and Environment 27: 1362-1373.
43. Schmidt DD, Voelckel C, Hartl M, Schmidt S, Baldwin IT (2005) Specificity in ecological interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. Plant Physiology 138: 1763-1773.
44. Saeed AI SV, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J (2003) TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34: 374-378.

Manuscript III

Why does herbivore attack reconfigure primary metabolism?

Jens Schwachtje and Ian T. Baldwin*

Accepted by Plant Physiology, invited review

Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Straße 8, D-07745 Jena, Germany
*Corresponding author: Ian T. Baldwin. Fax: +49-3641-571102. Email: baldwin@ice.mpg.de

Journal research area: Plants interacting with other organisms
Focus Issue: Plant Interaction with Arthropod Herbivores

Introduction

A plant's resistance to herbivore attack is thought to be principally determined by its secondary metabolism, which can be remarkably plastic and responsive to different grades and types of herbivory. Newer unbiased "omic" approaches, which characterize transcriptomic, metabolomic, and proteomic changes in herbivore-attacked plants, have laid to rest the notion that metabolism can be neatly parsed into "secondary metabolism", which functions to meet environmental challenges, and "primary metabolism", which supports growth. The hundreds of genes regulated during the plant-herbivore or -pathogen interaction have been analyzed with microarray studies, and almost all aspects of metabolism are represented, with a substantial fraction coming from primary metabolism (Hui et al., 2003; Major and Constabel, 2006; Mozoruk et al., 2006; Ralph et al., 2006; Schmidt and Baldwin, 2006; Kant and Baldwin, 2007; Both et al., 2005; Alignan et al., 2006; Tian et al., 2006).

Here we consider 4 overlapping functional explanations for this reconfiguration:

I. Resistance traits are **costly** and frequently up-regulated after attack, requiring reductions in growth, reproduction or storage and/or increases in assimilation to meet their metabolic demands (Fig. 1). These changes in resource allocation can be either *acute*, driven by immediate reductions in resources, or *anticipatory*, occuring before resource supply limits defense activation (Smith and Stitt, 2007).
II. Rather than supporting defense responses, reconfiguration could support the physiological adjustments plants must make to **tolerate** herbivory and reduce the negative fitness consequences of herbivore attack (Fig. 1).
III. Primary metabolites could function as **signals** in defense pathways (Fig. 2).
IV. Induced changes in primary metabolism could **themselves be defensive** (Fig. 2).

We consider these 4 hypotheses in an overview of the literature that addresses how assimilation and the partitioning of assimilates are altered by herbivory and how primary metabolites function as signals and as defenses. In conclusion, we consider the challenges that plant biologists face in attempting to falsify these hypotheses. Compared to the falsifications of hypotheses about the defensive function of secondary metabolism, tests of the above hypotheses will seriously challenge the procedures that we use to understand resistance mechanisms and perhaps even challenge the reductionist paradigm that has proved so useful for understanding gene function in much of biology in the last century.

Herbivore-induced changes in assimilation and partitioning of assimilates

If the resource demands of defense production compete with those of growth and reproduction, and defenses are costly to produce (Steppuhn, 2007), then changes in the partitioning of resources among growth, storage and reproduction would be expected; otherwise the resource demands of increased defense production would not be met (Fig. 1).

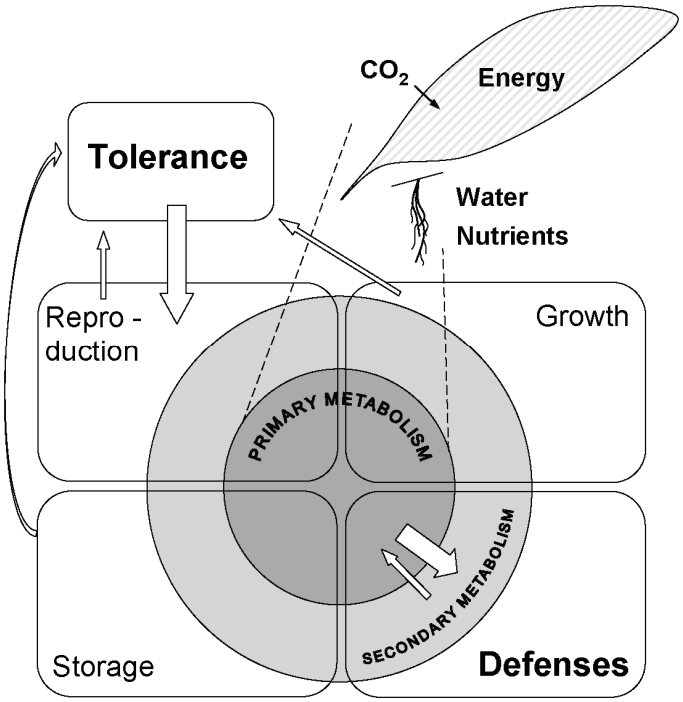


Fig. 1. Dependency of resistance traits (defenses and tolerance) and primary metabolism. Primary metabolism is fuelled by energy and resources, which the plant gains from its environment. Primary metabolism involves growth, storage and reproduction. Tolerance depends on primary metabolites and energy, both of which are taken from pools for reproduction, storage, and/or growth, and later re-invested in reproduction. Defenses from secondary metabolism are based on energy and resources from primary metabolism, which can be partially re-supplied to primary metabolism. Parts of primary metabolism can function as direct defensive.

Changes in how resources are partitioned according to function can be avoided if the rate of resource assimilation increases. Similar predictions hold for the activation of physiological changes that allow plants to better tolerate the negative fitness consequences of herbivory. Tolerance, which measures a plant's ability to compensate for the negative fitness effects of tissue damage, is usually described as a reaction norm of the fitness of specific genotypes at various damage levels (Strauss and Agrawal, 1999; Stowe et al., 2000). Tolerance is thought to result from the activation of dormant meristems, changes in plant

architecture, or photosynthetic capacity. In short, the herbivory-induced activation of both defense and tolerance responses is predicted to alter resource assimilation and source-sink relationships and the literature provides general support of these predictions.

The photosynthetic apparatus frequently responds to herbivore attack, usually with decreases in CO_2 assimilation in the attacked leaf that are proportionally greater than the leaf area that is actually damaged (Zangerl et al., 2002). Otherwise the photosynthetic response depends on the type of attacker and the age of the tissues that is measured (Welter, 1989). Defoliating herbivores can increases the photosynthetic activity of unattacked leaves, whereas stem borers and mesophyll feeders tend to decrease activity. On the other hand, transcript levels of photosynthetically related genes are commonly down-regulated (Hui et al., 2003; Ralph et al., 2006; Tang et al., 2006). It is thought that down-regulation of the photosynthetic apparatus protects it from oxidative damage (Niyogi, 2000), but decreased photosynthetic activity may also free up resources, especially nitrogen-rich compounds, making these available for use in secondary defense pathways. Decreased photosynthetic rates may be part of a global inhibition of protein synthesis, which may anticipate the need to redirect resources to defensive functions. As decreases in photosynthetic rates are more common than increases, altered photosynthesis has only rarely been correlated with tolerance (e.g. Cullen et al., 2006). Increases in photosynthetic rates could also be caused by changes in source-sink relationships resulting from the increased demand for energy and carbon-based resources that the production of defensive compounds entails; separating where and for what additional carbon and energy are used is difficult. The activation of dormant meristems and thus new sinks has been shown to be central in tolerance in some species (Bergelson et al., 1996; Mabry and Wayne, 1997). For example in *N. attenuata*, increased branching compensates for leaf damage (Schwachtje et al., 2006) and jasmonic acid (JA) signaling, which is responsible for activating several defense responses in this species, appears to suppress regrowth and contribute to apical dominance (Zavala and Baldwin, 2006).

How herbivore attack alters source/sink relationships remains unclear other than by reducing source strength when herbivores consume and damage leaves. As well as serving a variety of developmental functions, invertases are involved in the regulation of sink strength by cleaving sucrose into glucose and fructose, thereby altering the osmotic gradient of sucrose and leading to altered carbohydrate partitioning by turning specific tissues into metabolic sinks for carbohydrates (Roitsch and Gonzalez, 2004). Hence, invertases are often regulated after insect attack. For example, increased sink strength is elicited by JA treatment and gypsy moth (*Lymantria dispar*) feeding via the increased activity of cell wall invertases in the sink

leaves of hybrid *Populus deltoides* X *P. nigra* (Arnold and Schultz, 2002) and the wounding of leaves in *Solanum lycopersicum*, *Lycopersicum peruvianum* and *Pisum sativum* which increases the activity of soluble (vacuolar) and cell wall invertase in damaged leaves (Zhang et al., 1996; Ohyama et al., 1998). Root wounding was shown to induce vacuolar and cell wall invertase in *Beta vulgaris* (Rosenkranz et al., 2001). Changes in assimilate flux after herbivore attack may occur along the transport routes, where sugar transporters are involved in sucrose loading and unloading. Wounding is known to elicit a sucrose transporter, AtSUC3, in sieve elements of different sink tissues (Meyer et al., 2004), and a monosaccharide transporter, STP4, in *Arabidopsis thaliana* (Truernit et al., 1996).

Tolerance to herbivore attack can be afforded by changing when stored reserves are used, for example, those of root tissues. This strategy favors biennial or perennial species that normally accumulate reserves during their growing season for later growth during short-day periods (Wyka, 1999; Wise and Cummins, 2006). With nutrients stored in safe tissues, e.g. roots, plants have the possibility to regrow later in the growing season when the pressure from above-ground herbivores may have decreased. If plants are attacked by root herbivores, assimilates can be remobilized above ground. The highly tolerant *Centaurea maculosa* responds to root herbivory by the knapweed moth (*Agapeta zoegana*) by reducing its nitrogen (N) uptake but also shifting N to above-ground tissues (Newingham et al., 2007), suggesting that N allocation can be a determinant of tolerance. This idea is supported by the finding that after its leaves were clipped, the dwarf shrub *Indigofera spinosa* increased its root N uptake (Coughenour et al., 1990), and that *Quercus serrata* accumulates higher N levels in leaves (Takashima et al., 2004). Moreover, N allocation to roots has been observed after methyl-JA treatment of *Medicago sativa* (Meuriot et al., 2004).

Carbon is allocated to roots in response to leaf damage or herbivory in several species, for example after grasshopper damage to *Zea mays* (Holland et al., 1996) and *Panicum coloratum* (Dyer 1991), after the defoliation of *Lolium perenne* (Bazot et al., 2005) and of two C_4 perennial grasses (Briske et al., 1996), and after methyl-JA treatment of *Populus tremuloides* (Babst et al., 2005). Recently, a SnRK kinase has been found to regulate the re-allocation of photoassimilates in response to herbivory, facilitating a tolerance response (Fig. 2, Schwachtje et al., 2006). SnRK kinases are involved in regulating isoprenoid, amino acid, and especially carbohydrate metabolism (Halford and Paul, 2003). The ß-subunit of the kinase complex is rapidly down-regulated in the source leaves of *N. attenuata* after simulated attack by the tobacco hornworm (*Manduca sexta*), leading to 10 % more photoassimilate being partitioned to roots. The same effect was seen in JA-deficient asLOX plants, which are

silenced for the JA-biosynthetic enzyme lipoxygenase, making this response demonstrably independent of JA signaling. This rapid bunkering of carbon into root tissues is elicited when wounds are treated with fatty acid-amino acid conjugates (FACs) which are the insect-specific elicitors that activate most defense responses via the jasmonate cascade (Halitschke et al., 2003). At the end of its growing season, *N. attenuata* gains a measure of tolerance by re-using its additional root resources to prolong it flowering, leading to increased capsule production late in the season. Recently, wild type *Arabidopsis* plants overexpressing JA were observed to have reduced fitness but the same tolerance of defoliation, which is consistent with the idea that there is a JA-independent mechanism of tolerance (Cipollini, 2007).

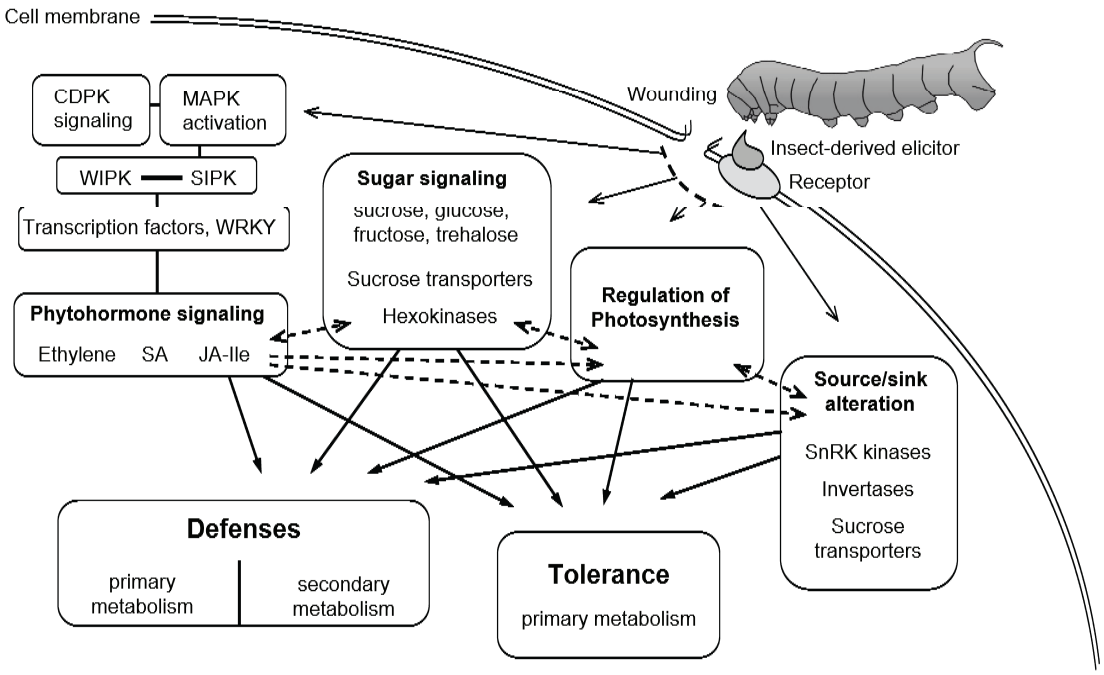


Fig. 2. Resistance signaling is elicited differently from simple wounding when herbivore-specific elicitors (FACs) are introduced into wounds during caterpillar feeding. Signaling depends on primary metabolites. Herbivory induces a large reorganization of primary metabolism, including altered photosynthesis and altered sink/source relations. These changes are coordinated by a signaling network that is only partially understood. The expression of defense and tolerance traits requires changes both in primary and secondary metabolism.

When *Manduca sexta* larvae attack its native host plant, *N. attenuata,* and FACs from the larvae's oral secretions are introduced into wounds during feeding, these elicit not only a suite of MAPKs and a complicated phytohormone response – which coordinate the increased

production of secondary metabolites that function as defense -- but also an SnRK kinase that increases the flux of carbon to the roots (Fig. 2). This increased flux of C to the roots would be expected to increase the rate of root growth, but in young seedlings, for example, sometimes just the opposite occurs (Hummel et al., 2007). Unlike the FAC-elicited C-flux, this rapid inhibition of root growth requires an intact JA signaling cascade (Hummel et al., in review) and may be one of the plant's anticipatory responses. Changes in growth that are anticipated in advance of resource limitations (and therefore differ from acute responses) have acquired growing importance as physiologists have shifted their focus to understanding the relationships between carbon balance and growth (Smith and Stitt, 2007).

By studying the growth dynamics of plants unable to synthesize starch due to a mutation in plastidial phosphoglucomutase (PGM) in combination with experimental conditions in which the dark cycle was extended, researchers have discovered that plants anticipate the length of the dark period and adjust their synthesis and catabolism of starch to exactly meet energy demands during the dark period (Gibon et al., 2004; Smith and Stitt, 2007). For reasons that are not completely clear, starch accumulation at the end of the dark period is inversely correlated with growth rate (Cross et al., 2006). It will be interesting to see how these anticipatory changes in allocation and resource partitioning that are likely coordinated by a plant's circadian clock are modified when plants are elicited by insect-specific elicitors.

Primary metabolites as signals and defenses

A plant's resistance response to insect feeding is coordinated by different signaling pathways that depend on primary metabolites; in addition, the integration of the different signals induced by wounding and insect-specific elicitors results in a complex rearrangement of primary and secondary metabolism (Fig. 2). JA is a crucial player in defense signaling (Devoto and Turner, 2005) and requires kinases, such as WIPK and SIPK (Wu et al., 2007), and transcription factors such as WRKYs (Hui et al., 2003). After elicitation, isoleucine (Ile) production is amplified by threonine deaminase(TD). Two hours after elicitation, the mRNA levels of *N. attenuata*'s TD are increased by as much as 30 times (Kang et al., 2006). The Ile that is produced at the attack site is rapidly conjugated to JA, forming JA-Ile, a key activator of defense signaling (Chini et al., 2007; Thines et al., 2007). In addition to the JA-dependent signaling, several JA-independent responses to wounding and FACs have been documented (Leon et al., 1998; Rojo et al., 1999; LeBrasseur et al., 2002; Gross et al., 2004; Schwachtje et al., 2006), but knowledge about the underlying mechanisms is limited. Recently, the signaling

role of sugars has received increased attention because several sugar-induced resistance genes have been found. For example, sucrose, glucose and fructose act as specific regulatory signals on the wound-inducible expression of an extensin gene (SbHRGP3) in *Glycine max* (Ahn et al., 1996; Ahn and Lee, 2003); additionally, a putatively defensive vegetative storage protein is sucrose- as well as JA-induced (Berger et al., 1995). Moreover, transcripts of a hexokinase, which can function as a sugar sensor or photosynthesis repressor (Rolland et al., 2006), are induced by wounding and are sensitive to trehalose-6-phosphate (Claeyssen and Rivoal, 2007), which itself is involved in the feedback regulation of photosynthesis and developmental transitions (Paul, 2007; Ramon and Rolland, 2007). Trehalose and SnRK protein kinases have been shown to interact (Schluepmann et al., 2004), as have sugars and lectins, which also can be induced by JA, suggesting lectins play a role in signal transduction (Chen et al., 2002; Van Damme et al., 2003; Gabius et al., 2004; Lannoo et al., 2006). Furthermore, an antagonistic interaction between glucose and ethylene, which is involved in defense signaling (von Dahl and Baldwin, 2007), has been reported (Zhou et al., 1998).

Defense signaling can also be elicited by non-self recognition of a primary metabolite (Schmelz et al., 2006; Schmelz et al., 2007). When V*igna unguiculata* is eaten by the fall armyworm (*Spodoptera frugiperda*), the larvae's gut proteases digest ingested chloroplastic ATPase, and fragments of the chloroplastic ATPase, christened inceptins, elicit JA, salicylic acid, and ethylene signaling when they are re-introduced into the plant during feeding .

Several metabolites that play well-studied roles in primary metabolism have been found to possess defensive functions. Their dual function has been discovered because very high levels of them accumulate in plants, or because their induction patterns after herbivore attack is similar to that of defensive secondary metabolites. In the case of TD, for example, the function of the enzyme, degrading threonine, led to the hypothesis that it functioned in the insect's gut to degrade this essential amino acid. TD's regulatory domain was found to be removed by insect proteases, suppressing its negative feedback regulation by Ile (Chen et al., 2007). TD then continuously degrades threonine in the gut lumen, leading to amino acid starvation. Two TD isoforms are known in *S. lycopersicum*, one of which is stable in insect guts (Chen et al., 2007); in *N. attenuata*, in contrast, one TD serves both primary and secondary functions (Kang et al., 2006).

High levels of calcium oxalate (CaOx), a primary metabolite, accumulate in plants (up to 80% of dry mass), and in some plants, CaOx synthesis is induced by herbivory (Molano-Flores, 2001; Ruiz et al., 2002). CaOx regulates bulk levels of the Ca that is involved in cell signaling and in several biochemical processes. The morphologically diverse CaOx crystals

are either stored in the vacuoles of specialized cells, the crystal idioblasts, or are associated with the cell wall (Franceschi and Nakata, 2005). Crystals can be located around tissues, e.g. vascular bundles, to provide a physical barrier against chewing insects by an abrasive effect that blunts insects' mandibles (Korth et al., 2006). Moreover, CaOx is thought to act as an antinutritive defense by decreasing the efficiency with which ingested food is converted (Korth et al., 2006).

Vegetative storage proteins (VSP) can make up large portions (40%) of soluble protein (Andrews et al., 1988). Long thought to serve only their annotated function, recently they have been recognized as potent defenses. In primary metabolism, VSPs may serve as transient storage reserves for reduced N (Staswick et al., 1994); but when they are silenced, they do not impair seed production (Staswick et al., 2001). Direct anti-insect functions have been attributed to them which correlate with acid phosphatase activity (Berger et al., 1995; Liu et al., 2005), trypsin inhibitory activity (sweet potato sporamin, (Yeh et al., 1997), chitinase activity (Meuriot et al., 2004), and acyl hydrolase activity (potato patatin; (Strickland et al., 1995) of the VSPs. Moreover, VSPs may be involved in re-shaping source/sink relations by generating N-demand where they are produced (Cooke and Weih, 2005).

Another group of proteins, lectins, can accumulate to relatively high concentrations (~ 10 %) in seeds and specialized vegetative tissues (Law and Kfir, 1997; Murdock and Shade, 2002; Van Damme et al., 2003). Although they may function as storage proteins to support vegetative growth (Rudiger and Gabius, 2001; Van Damme et al., 2003), they also act defensively. They are divided into seven related families, some of which are ubiquitous in plants (Van Damme et al., 1998). Most lectins have a chitin- or sugar-binding capacity (Yunes et al., 1998; Murdock and Shade, 2002). A well-studied defensive lectin is a mannose-binding lectin from *Galanthus nivalis*, which strongly increases the mortality of both a leafhopper (*Empoasca fabae*) and a scarab beetle (*Antitrogus parvulus*), as well as the growth of a planthopper (*Nilaparvata lugens*) when added to artificial diet at 0.2-6 µM (Habibi et al., 1993; Allsopp and McGhie, 1996; Powell et al., 1998). Lectins are thought to bind to the chemosensory apparatus of the herbivore, where they block food chemistry signals, disturb the formation of the peritrophic matrix, and damage the hemolymph and ovaries (Powell et al., 1998). Interestingly, stress-inducible lectin genes are assumed to be ancestral because homologues have been found in lower plants, e.g. ferns; their function as storage proteins is assumed to have evolved as a secondary trait (Van Damme et al., 2003).

Single amino acids can also act defensively. Several proteinogenic amino acids have been shown to negatively influence larval development if added in quantities greater than 5 %

of an artificial diet (Janzen et al., 1977). For example, L-tyrosine that hyperaccumulates in *Inga umbellifera* in quantities constituting up to 10% of dry mass of young leaves, dramatically reduced the growth of the tobacco budworm (*Heliothis virescens*) in feeding assays (Lokvam et al., 2006). The non-protein amino acid γ-aminobutyrate (GABA) has several primary functions in plants: in the GABA gradient-driven direction of pollen tube growth towards the micropyle (Palanivelu et al., 2003), in pH control and N storage (Shelp et al., 1999), and in regulating 14-3-3 proteins (Lancien and Roberts, 2006). GABA, is induced by the wounding resulting from insect movement on leaf surfaces (Ramputh and Bown, 1996; Bown et al., 2002), suggesting a role in stress signaling (Bouche et al., 2003). GABA deters feeding by the tobacco budworm (MacGregor et al., 2003) and decreases growth of a leaf-roller larva (*Choristoneura rosaceana*) (Ramputh and Bown, 1996). In invertebrates, GABA is an inhibitory neurotransmitter and can cause paralysis by disturbing GABA-gated Cl^--channels.

Carbohydrates can also directly function as defenses. Galactose markedly reduced larval growth of western spruce budworm (*Choristoneura occidentalis*) when added to artificial diet in quantities of 6%, but glucose and fructose increased growth (Zou and Cates, 1994). In *Acacia*, mutualistic ants are attracted by nectar sugars (Heil et al., 2005). However, only after sucrose is cleaved by an invertase, which is present in extrafloral nectar, are nonsymbiotic ants repelled and mutualistic ants attracted.

Testing hypotheses about defensive function

Whether a given secondary metabolite plays a role in herbivore protection is best determined by planting isogenic plants that both do and do not produce the metabolite into the plants' native environment where they can be confronted with native herbivore communities. With the development of transformation systems and the identification of genes that control the biosynthesis and flux into secondary metabolism, it is now possible to create these isogenic plants, and to test their function. For example nicotine-, TPI- and JA-signaling-deficient *N. attenuata* plants have provided strong proof for the defensive function of individual secondary metabolites, for defensive synergies among different secondary metabolites, and for the role of JA signaling in activating metabolic changes (Kessler et al., 2004; Steppuhn et al., 2004; Zavala et al., 2004; Steppuhn and Baldwin, 2007).

The defensive metabolites of a plant have long been thought to act synergistically: the combination of different effects is assumed to be more than their parts. A defensive synergism between nicotine and TPIs was discovered in *N. attenuata* when the production of nicotine or

TPI or both was silenced, or when plants were attacked by the second most common lepidopteran herbivore in this tobacco's natural habitat, *Spodoptera exigua.* The compensatory feeding response of this herbivore to TPI-induced amino acid starvation was inhibited by the larvae's limited ability to tolerate nicotine and the leaf area consumed was reduced when both secondary metabolites were present (Steppuhn and Baldwin, 2007). Similarly artificial diet experiments showed that plant-derived phytic acid, a primary metabolite, reduces the detoxification of xanthotoxin, a defensive furanocoumarin, by the parsnip webworm (*Depressaria pastinacella*), likely by inhibiting insect CYP450 monoxygenases (Green et al., 2001). These results demonstrate that *in planta* tests are essential for pinpointing defensive function, because the chemical milieu in which a metabolite is expressed can profoundly influence how an herbivore responds.

These secondary metabolite-deficient plants also provided strong support for the hypothesis that secondary metabolites -- previously thought to be directed solely at agents outside the plant – play a physiological role inside the plant. Silencing a trypsin protease inhibitor (TPI) gene not only increased plants' susceptibility to herbivores but increased growth and seed production. This increase in plant fitness, apparent not only when TPI production was silenced but also when TPI production was restored in an ecotype naturally deficient in TPI production (Zavala et al., 2004; Zavala et al., 2004), is not likely explained by the liberation of resources that are no longer invested in TPI production. While the mechanisms remain to be worked out, it is likely that TPIs down-regulate growth through their modulation of a yet-to-be described signaling cascade.

Molecular biology made it possible to uncover the defensive functions of secondary metabolites, because it was possible to silence the accumulation of a secondary metabolite with simple RNAi constructs driven by constitutive promotors without simultaneously affecting plant growth. Tests of the defensive function of primary metabolites will require subtle silencing tools that allow silencing to be both tissue specific and controlled at very precise times: the goal is to minimize the growth and developmental effects of gene silencing while determining herbivore performance and resistance under native conditions. Integrative approaches that compare the effects of gene silencing at different levels in the signaling hierarchy will be necessary to determine how resources are allocated and source/sink relations adjusted. Asking the proteome, metabolome and transcriptome for answers using unbiased analytical tools will, we hope, identify those regulatory nodes that are altered by herbivore attack. While (ultra) high-throughput analytical and data handling platforms will be important for handling the torrent of data produced, student training that emphasizes real-world

familiarity with the plant and its natural history will also be important. Students will need to be trained to use an experimental approach that inverts the normal sequence of events in the biological discovery process (Fig. 3). Instead of proceeding step-by-step from gene, to transcript, protein, metabolite, glasshouse phenotype and only when the plants are fully characterized, to a field test, field tests will need to be carried out earlier in the analysis. In this way, biological intuition will again become a valued trait among plant biologists.

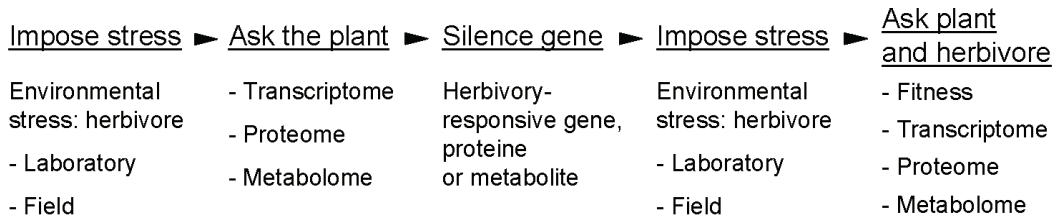


Fig. 3. The study of resistance traits from primary metabolism by gene silencing requires an integrative approach based on several techniques. Environmental stimuli together with the blend of "omics"-techniques are crucial to determine herbivore-responsive genes, proteins or metabolites from primary metabolism. Silencing the associated gene(s) reveals fitness-relevant functions in field and lab studies, and "omics" techniques uncover their influence on primary and secondary metabolism.

The discovery of the function of *N. attenuata*'s RNA-direct RNA polymerases (RdR1 and 2) in mediating resistance responses to herbivore attack and UV-B illustrates the procedure (Pandey and Baldwin, in review; Pandey and Baldwin, 2007). RdRs are essential in siRNA biogenesis but their organismic-level function was unknown. When plants silenced in endogenous RdR1 expression were planted into native populations, they were found to highly susceptibility to attack from native herbivores, which was associated with reduced nicotine levels, which in turn are known to require JA signaling for their upregulation. Further analysis revealed that RdR1 was involved in coordinating the signaling of phytohormones, namely ethylene and JA. Silencing RdR2, on the other hand, resulted in reduced growth and reproductive performance in the field, but their resistance to the native herbivores and pathogens was unaffected. However, when competed against WT plants in the glasshouse, their growth and reproductive performance was found not to differ from WT plants. From these results we deduced that the RdR2-silenced plants were susceptible to UV-B and which was subsequently confirmed in glasshouse experiments and traced to deficiencies in the production of phenolic UV-B sunscreens. These examples demonstrate that field experiments are indispensable for unraveling ecological gene functions that are overlooked when plants are grown in protected glasshouse environments and that an intimate understanding of an

organism's natural history is essential for interpreting new phenotypes caused by silencing of primary genes.

Heterotrophy was well established well before the evolution of photosynthesis. Plants have always had to cope with the ravages of consumers that want access to the resources that plants control. Their sophisticated means of defending themselves likely use all aspects of their metabolism. Their prodigious anabolic potential allows plants to throw just about everything at consumers to protect themselves. Our challenge will be figure out what parts of metabolism are currently being maintained by natural selection as defenses. As Rick Karban predicted in his "Moving Target Hypothesis" (Karban et al., 1997), plants are very plastic and this plasticity itself may well be part of its defensive repertoire.

Although nature provides the best laboratory for testing gene function, we have trained a generation of plant biologists who are unfamiliar with field work. When unbiased transcriptional responses are used to "ask the plant" which genes are regulated in response to herbivore attack, the plant provides testable hypotheses about which genes are important in tolerance or defense. Gene annotations classify genes and specify their putative biochemical function based on sequence similarity. These annotations are extremely valuable but they should be viewed with caution as they do not exclude other biochemical functions or functions at other levels. An example of the TD gene from *N. attenuata* illustrates the point. Silencing TD generated plants with stunted growth, because TD is involved in Ile biosynthesis. However, other TD-silenced plants grew normally but were found to be highly susceptible to herbivores (Kang et al., 2006). Further analysis of these plants revealed a strong reduction of defense responses to herbivory, due to reduced levels of JA-Ile: defense signaling was hampered, but the effects on primary metabolism were negligible. This illustrates that the defense function of a primary gene can be studied with transformants that exhibit "mild" phenotypes and are not impaired in development.

Clearly there will be much to be learned by "asking the plant" and using the "omic" tools for deciphering the plant's answer in the genes, proteins and metabolites that it regulates differently when attacked by herbivores. If we are sufficiently forward thinking to ignore the gene annotations, to silence these regulated responses in ways that don't dramatically influence growth and then to ask the community of herbivores that naturally attack plants whether the plant is more resistant to or tolerant of herbivore attack, we will undoubtedly learn much that is new about how plants survive in the real world (Fig 3). Although at present technical and regulatory issues impede the adoption of this procedure, the blinders that come with specialized scientific training will be as difficult to remove as the other challenges.

Literature Cited

Ahn JH, Choi Y, Kwon YM, Kim SG, Choi YD, Lee JS (1996) A novel extensin gene encoding a hydroxyproline-rich glycoprotein requires sucrose for its wound-inducible expression in transgenic plants. Plant Cell **8:** 1477-1490

Ahn JH, Lee JS (2003) Sugar acts as a regulatory signal on the wound-inducible expression of SbHRGP3 :: GUS in transgenic plants. Plant Cell Reports **22:** 286-293

Alignan M, Hewezi T, Petitprez M, Dechamp-Guillaume G, Gentzbittel L (2006) A cDNA microarray approach to decipher sunflower (*Helianthus annuus*) responses to the necrotrophic fungus *Phoma macdonaldii*. New Phytologist **170:** 523-536

Allsopp PG, McGhie TK (1996) Snowdrop and wheatgerm lectins and avidin as antimetabolites for the control of sugarcane whitegrubs. Entomologia Experimentalis Et Applicata **80:** 409-414

Andrews DL, Beames B, Summers MD, Park WD (1988) Characterization of the lipid acyl hydrolase activity of the major potato (*Solanum tuberosum*) tuber protein, patatin, by cloning and abundant expression in a baculovirus vector. Biochemical Journal **252:** 199-206

Arnold TM, Schultz JC (2002) Induced sink strength as a prerequisite for induced tannin biosynthesis in developing leaves of *Populus*. Oecologia **130:** 585-593

Babst BA, Ferrieri RA, Gray DW, Lerdau M, Schlyer DJ, Schueller M, Thorpe MR, Orians CM (2005) Jasmonic acid induces rapid changes in carbon transport and partitioning in *Populus*. New Phytologist **167:** 63-72

Bazot S, Mikola J, Nguyen C, Robin C (2005) Defoliation-induced changes in carbon allocation and root soluble carbon concentration in field-grown *Lolium perenne* plants: do they affect carbon availability, microbes and animal trophic groups in soil? Functional Ecology **19:** 886-896

Bergelson J, Juenger T, Crawley MJ (1996) Regrowth following herbivory in *Ipomopsis aggregata*: Compensation but not overcompensation. American Naturalist **148:** 744-755

Berger S, Bell E, Sadka A, Mullet JE (1995) *Arabidopsis thaliana* Atvsp is homologous to soybean Vspa and Vspb, genes encoding vegetative storage protein acid-phosphatases, and is regulated similarly by methyl jasmonate, wounding, sugars, light and phosphate. Plant Molecular Biology **27:** 933-942

Both M, Csukai M, Stumpf MPH, Spanu PD (2005) Gene expression profiles of *Blumeria graminis* indicate dynamic changes to primary metabolism during development of an obligate biotrophic pathogen. Plant Cell **17:** 2107-2122

Bouche N, Lacombe B, Fromm H (2003) GABA signaling: a conserved and ubiquitous mechanism. Trends in Cell Biology **13:** 607-610

Bown AW, Hall DE, MacGregor KB (2002) Insect footsteps on leaves stimulate the accumulation of 4-aminobutyrate and can be visualized through increased chlorophyll fluorescence and superoxide production. Plant Physiology **129:** 1430-1434

Briske DD, Boutton TW, Wang Z (1996) Contribution of flexible allocation priorities to herbivory tolerance in C-4 perennial grasses: An evaluation with C-13 labeling. Oecologia **105:** 151-159

Chen H, Gonzales-Vigil E, Wilkerson CG, Howe GA (2007) Stability of plant defense proteins in the gut of insect herbivores. Plant Physiology **143:** 1954-1967

Chen Y, Peumans WJ, Hause B, Bras J, Kumar M, Proost P, Barre A, Rouge P, Van Damme EJM (2002) Jasmonate methyl ester induces the synthesis of a cytoplasmic/nuclear chitooligosaccharide-binding lectin in tobacco leaves. Faseb Journal **16:** 905-931

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666-673

Cipollini D (2007) Consequences of the overproduction of methyl jasmonate on seed production, tolerance to defoliation and competitive effect and response of *Arabidopsis thaliana*. New Phytologist **173:** 146-153

Claeyssen E, Rivoal J (2007) Isozymes of plant hexokinase: occurrence, properties and functions. Phytochemistry **68:** 709-731

Cooke JEK, Weih M (2005) Nitrogen storage and seasonal nitrogen cycling in *Populus*: bridging molecular physiology and ecophysiology. New Phytologist **167:** 19-30

Coughenour MB, Detling JK, Bamberg IE, Mugambi MM (1990) Production and nitrogen responses of the african dwarf shrub *Indigofera spinosa* to defoliation and water limitation. Oecologia **83:** 546-552

Cross JM, von Korff M, Altmann T, Bartzetko L, Sulpice R, Gibon Y, Palacios N, Stitt M (2006) Variation of enzyme activities and metabolite levels in 24 *Arabidopsis* accessions growing in carbon-limited conditions. Plant Physiology **142:** 1574-1588

Cullen BR, Chapman DF, Quigley PE (2006) Comparative defoliation tolerance of temperate perennial grasses. Grass and Forage Science **61:** 405-412

Devoto A, Turner JG (2005) Jasmonate-regulated *Arabidopsis* stress signalling network. Physiologia Plantarum **123:** 161-172

Franceschi VR, Nakata PA (2005) Calcium oxalate in plants: formation and function. Annual Review of Plant Biology **56:** 41-71

Gabius HJ, Siebert HC, Andre S, Jimenez-Barbero J, Rudiger H (2004) Chemical biology of the sugar code. Chembiochem **5:** 741-764

Gibon Y, Blasing O, Palacios-Rojas N, Pankovic D, Hendriks J, Fisahn J, Hohne M, Gunther, M S (2004) Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post-translational activation of ADP-glucose pyrophosphorylase in the following light period. Plant Journal **39:** 847-862

Green ES, Zangerl AR, Berenbaum MR (2001) Effects of phytic acid and xanthotoxin on growth and detoxification in caterpillars. Journal of Chemical Ecology **27:** 1763-1773

Gross N, Wasternack C, Kock M (2004) Wound-induced RNaseLE expression is jasmonate and systemin independent and occurs only locally in tomato (*Lycopersicon esculentum* cv. Lukullus). Phytochemistry **65:** 1343-1350

Habibi J, Backus EA, Czapla TH (1993) Plant-lectins affect survival of the potato leafhopper (Homoptera, Cicadellidae). Journal of Economic Entomology **86:** 945-951

Halford NG, Paul MJ (2003) Carbon metabolite sensing and signalling. Plant Biotechnology Journal **1:** 381-398

Halitschke R, Gase K, Hui D, Schmidt DD, Baldwin IT (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. Plant Physiology **131:** 1894-1902

Heil M, Rattke J, Boland W (2005) Postsecretory hydrolysis of nectar sucrose and specialization in ant/plant mutualism. Science **308:** 560-563

Holland JN, Cheng WX, Crossley DA (1996) Herbivore-induced changes in plant carbon allocation: assessment of below-ground C fluxes using carbon-14. Oecologia **107:** 87-94

Hui DQ, Iqbal J, Lehmann K, Gase K, Saluz HP, Baldwin IT (2003) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*: V. Microarray analysis and

further characterization of large-scale changes in herbivore-induced mRNAs. Plant Physiology **131:** 1877-1893

Hummel GM, Naumann M, Schurr U, Walter A (2007) Root growth dynamics of *Nicotiana attenuata* seedlings are affected by herbivory. Plant Cell Environment **30:** 1326-1336

Janzen DH, Juster HB, Bell EA (1977) Toxicity of secondary compounds to seed-eating larvae of bruchid beetle *Callosobruchus maculatus*. Phytochemistry **16:** 223-227

Kang JH, Wang L, Giri A, Baldwin IT (2006) Silencing threonine deaminase and JAR4 in *Nicotiana attenuata* impairs jasmonic acid-isoleucine-mediated defenses against *Manduca sexta*. Plant Cell **18:** 3303-3320

Kant M, Baldwin I (2007) The ecogenetics and ecogenomics of plant-herbivore interactions: rapid progress on a slippery. Current Opinion in Genetics and Development **17:** xxx-xxx

Karban R, Agrawal AA, Mangel M (1997) The benefits of induced defenses against herbivores. Ecology **78:** 1351-1355

Kessler A, Halitschke R, Baldwin IT (2004) Silencing the jasmonate cascade: Induced plant defenses and insect populations. Science **305:** 665-668

Korth KL, Doege SJ, Park SH, Goggin FL, Wang Q, Gomez SK, Liu G, Jia L, Nakata PA (2006) *Medicago truncatula* mutants demonstrate the role of plant calcium oxalate crystals as an effective defense against chewing insects. Plant Physiology **141:** 188-195

Lancien M, Roberts MR (2006) Regulation of *Arabidopsis thaliana* 14-3-3 gene expression by gamma-aminobutyric acid. Plant Cell and Environment **29:** 1430-1436

Lannoo N, Peumans WJ, Van Pamel E, Alvarez R, Xiong TC, Hause G, Mazars C, Van Damme EJM (2006) Localization and in vitro binding studies suggest that the cytoplasmic/nuclear tobacco lectin can interact in situ with high-mannose and complex N-glycans. FEBS Letters **580:** 6329-6337

Law IJ, Kfir R (1997) Effect of mannose-binding lectin from peanut and pea on the stem borer *Chilo partellus*. Entomologia Experimentalis Et Applicata **82:** 261-265

LeBrasseur ND, MacIntosh GC, Perez-Amador MA, Saitoh M, Green PJ (2002) Local and systemic wound-induction of RNase and nuclease activities in *Arabidopsis*: RNS1 as a marker for a JA-independent systemic signaling pathway. Plant Journal **29:** 393-403

Leon J, Rojo E, Titarenko E, Sanchez-Serrano JJ (1998) Jasmonic acid-dependent and -independent wound signal transduction pathways are differentially regulated by Ca2+/calmodulin in *Arabidopsis thaliana*. Molecular and General Genetics **258:** 412-419

Liu YL, Ahn JE, Datta S, Salzman RA, Moon J, Huyghues-Despointes B, Pittendrigh B, Murdock LL, Koiwa H, Zhu-Salzman K (2005) *Arabidopsis* vegetative storage protein is an anti-insect acid phosphatase. Plant Physiology **139:** 1545-1556

Lokvam J, Brenes-Arguedas T, Lee JS, Coley PD, Kursar TA (2006) Allelochemic function for a primary metabolite: the case of L-tyrosine hyper-production in *Inga umbellifera* (Fabaceae). American Journal of Botany **93:** 1109-1115

Mabry CM, Wayne PW (1997) Defoliation of the annual herb *Abutilon theophrasti*: mechanisms underlying reproductive compensation. Oecologia **111:** 225-232

MacGregor KB, Shelp BJ, Peiris S, Bown AW (2003) Overexpression of glutamate decarboxylase in transgenic tobacco plants deters feeding by phytophagous insect larvae. Journal of Chemical Ecology **29:** 2177-2182

Major IT, Constabel CP (2006) Molecular analysis of poplar defense against herbivory: comparison of wound- and insect elicitor-induced gene expression. New Phytologist **172:** 617-635

Meuriot F, Noquet C, Avice JC, Volenec JJ, Cunningham SM, Sors TG, Caillot S, Ourry A (2004) Methyl jasmonate alters N partitioning, N reserves accumulation and induces gene expression of a 32-kDa vegetative storage protein that possesses chitinase activity in *Medicago sativa* taproots. Physiologia Plantarum **120:** 113-123

Meyer S, Lauterbach C, Niedermeier M, Barth I, Sjolund RD, Sauer N (2004) Wounding enhances expression of AtSUC3, a sucrose transporter from *Arabidopsis* sieve elements and sink tissues. Plant Physiology **134:** 684-693

Molano-Flores B (2001) Herbivory and calcium concentrations affect calcium oxalate crystal formation in leaves of Sida (Malvaceae). Annals of Botany **88:** 387-391

Mozoruk J, Hunnicutt LE, Cave RD, Hunter WB, Bausher MG (2006) Profiling transcriptional changes in *Citrus sinensis* (L.) Osbeck challenged by herbivory from the xylem-feeding leafhopper *Homalodisca coagulata* (Say) by cDNA macroarray analysis. Plant Science **170:** 1068-1080

Murdock LL, Shade RE (2002) Lectins and protease inhibitors as plant defenses against insects. Journal of Agricultural and Food Chemistry **50:** 6605-6611

Newingham BA, Callaway RM, BassiriRad H (2007) Allocating nitrogen away from a herbivore: a novel compensatory response to root herbivory. Oecologia **153:** 913-920

Niyogi KK (2000) Safety valves for photosynthesis. Current Opinion in Plant Biology **3:** 455-460

Ohyama A, Nishimura S, Hirai M (1998) Cloning of cDNA for a cell wall-bound acid invertase from tomato (*Lycopersicon esculentum*) and expression of soluble and cell wall-bound invertases in plants and wounded leaves of *L. esculentum* and *L. peruvianum*. Genes & Genetic Systems **73:** 149-157

Palanivelu R, Brass L, Edlund AF, Preuss D (2003) Pollen tube growth and guidance is regulated by POP2, an *Arabidopsis* gene that controls GABA levels. Cell **114:** 47-59

Pandey S, Baldwin IT (200x) Silencing RNA-directed RNA polymerase 2 (RdR2) increases *Nicotiana attenuata*'s susceptibility to UV in the field and in the glasshouse. submitted **xx:** xxxx-xxxx

Pandey SP, Baldwin IT (2007) RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of Nicotiana attenuata to herbivore attack in nature. Plant Journal **50:** 40-53

Paul M (2007) Trehalose 6-phosphate. Current Opinion in Plant Biology **10:** 303-309

Powell KS, Spence J, Bharathi M, Gatehouse JA, Gatehouse AMR (1998) Immunohistochemical and developmental studies to elucidate the mechanism of action of the snowdrop lectin on the rice brown planthopper, *Nilaparvata lugens* (Stal). Journal of Insect Physiology **44:** 529-539

Ralph SG, Yueh H, Friedmann M, Aeschliman D, Zeznik JA, Nelson CC, Butterfield YSN, Kirkpatrick R, Liu J, Jones SJM, Marra MA, Douglas CJ, Ritland K, Bohlmann J (2006) Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. Plant Cell and Environment **29:** 1545-1570

Ramon M, Rolland F (2007) Plant development: introducing trehalose metabolism. Trends in Plant Science **12:** 185-188

Ramputh AL, Bown AW (1996) Rapid gamma-aminobutyric acid synthesis and the inhibition of the growth and development of oblique-banded leaf-roller larvae. Plant Physiology **111:** 1349-1352

Roitsch T, Gonzalez MC (2004) Function and regulation of plant invertases: sweet sensations. Trends in Plant Science **9:** 606-613

Rojo E, Leon J, Sanchez-Serrano JJ (1999) Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*. Plant Journal **20:** 135-142

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: Conserved and novel mechanisms. Annual Review of Plant Biology **57:** 675-709

Rosenkranz H, Vogel R, Greiner S, Rausch T (2001) In wounded sugar beet (*Beta vulgaris* L.) tap-root, hexose accumulation correlates with the induction of a vacuolar invertase isoform. Journal of Experimental Botany **52:** 2381-2385

Rudiger H, Gabius HJ (2001) Plant lectins: occurrence, biochemistry, functions and applications. Glycoconjugate Journal **18:** 589-613

Ruiz N, Ward D, Saltz D (2002) Calcium oxalate crystals in leaves of *Pancratium sickenbergeri*: constitutive or induced defence? Functional Ecology **16:** 99-105

Schluepmann H, van Dijken A, Aghdasi M, Wobbes B, Paul M, Smeekens S (2004) Trehalose mediated growth inhibition of *Arabidopsis* seedlings is due to trehalose-6-phosphate accumulation. Plant Physiology **135:** 879-890

Schmelz EA, Carroll MJ, LeClere S, Phipps SM, Meredith J, Chourey PS, Alborn HT, Teal PEA (2006) Fragments of ATP synthase mediate plant perception of insect attack. Proceedings of the National Academy of Sciences of the United States of America **103:** 8894-8899

Schmelz EA, LeClere S, Carroll MJ, Alborn HT, Teal PEA (2007) Cowpea chloroplastic ATP synthase is the source of multiple plant defense elicitors during insect herbivory. Plant Physiology **144:** 793-805

Schmidt DD, Baldwin IT (2006) Transcriptional responses of *Solanum nigrum* to methyl jasmonate and competition: a glasshouse and field study. Functional Ecology **20:** 500-508

Schwachtje J, Minchin PEH, Jahnke S, van Dongen JT, Schittko U, Baldwin IT (2006) SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. Proceedings of the National Academy of Sciences of the United States of America **103:** 12935-12940

Shelp BJ, Bown AW, McLean MD (1999) Metabolism and functions of gamma-aminobutyric acid. Trends in Plant Science **4:** 446-452

Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. Plant Cell and Environment **30:** 1126-1149

Staswick P, Zhang Z, 'Clemente T, Specht J (2001) Efficient down-regulation of the major vegetative storage protein genes in transgenic soybean does not compromise plant productivity. Plant Physiology **127** 1819-1826

Staswick PE, Papa C, Huang JF, Rhee Y (1994) Purification of the major soybean leaf acid-phosphatase that is increased by seed-pod removal. Plant Physiology **104:** 49-57

Steppuhn A (2007) Costs and benefits of two direct defenses in *Nicotiana attenuata*: nicotine and trypsin protease inhibitors. PhD thesis. Friedrich-Schiller-University, Jena

Steppuhn A, Baldwin IT (2007) Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. Ecology Letters **10:** 499-511

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. PloS Biology **2:** 1074-1080

Stowe KA, Marquis RJ, Hochwender CG, Simms EL (2000) The evolutionary ecology of tolerance to consumer damage. Annual Review of Ecology and Systematics **31:** 565-595

Strauss SY, Agrawal AA (1999) The ecology and evolution of plant tolerance to herbivory. Trends in Ecology & Evolution **14:** 179-185

Strickland JA, Orr GL, Walsh TA (1995) Inhibition of *Diabrotica* larval growth by patatin, the lipid acyl hydrolase from potato-tubers. Plant Physiology **109:** 667-674

Takashima T, Hikosaka K, Hirose T (2004) Photosynthesis or persistence: nitrogen allocation in leaves of evergreen and deciduous *Quercus* species. Plant Cell and Environment **27:** 1047-1054

Tang JY, Zielinski RE, Zangerl AR, Crofts AR, Berenbaum MR, DeLucia EH (2006) The differential effects of herbivory by first and fourth instars of *Trichoplusia ni* (Lepidoptera : Noctuidae) on photosynthesis in *Arabidopsis thaliana*. Journal of Experimental Botany **57:** 527-536

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu GH, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCFCO11 complex during jasmonate signalling. Nature **448:** 661-665

Tian ZD, Liu J, Wang BL, Xie CH (2006) Screening and expression analysis of *Phytophthora infestans* induced genes in potato leaves with horizontal resistance. Plant Cell Reports **25:** 1094-1103

Truernit E, Schmid J, Epple P, Illig J, Sauer N (1996) The sink-specific and stress-regulated *Arabidopsis* STP4 gene: Enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors, and pathogen challenge. Plant Cell **8:** 2169-2182

Van Damme EJM, Lannoo N, Fouquaert E, Peumans WJ (2003) The identification of inducible cytoplasmic/nuclear carbohydrate-binding proteins urges to develop novel concepts about the role of plant lectins. Glycoconjugate Journal **20:** 449-460

Van Damme EJM, Peumans WJ, Barre A, Rouge P (1998) Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Critical Reviews in Plant Sciences **17:** 575-692

von Dahl CC, Baldwin IT (2007) Deciphering the role of ethylene in plant-herbivore interactions. Journal of Plant Growth Regulation **26:** 201-209

Welter SC (1989) Arthropod impact on plant gas exchange. *In* EA Bernays, ed, Insect-plant interactions, Vol 1. CRC Press, Boca Raton, pp 135-150

Wise MJ, Cummins JJ (2006) Strategies of *Solanum carolinense* for regulating maternal investment in response to foliar and floral herbivory. Journal of Ecology **94:** 629-636

Wu JQ, Hettenhausen C, Meldau S, Baldwin IT (2007) Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*. Plant Cell **19:** 1096-1122

Wyka T (1999) Carbohydrate storage and use in an alpine population of the perennial herb, *Oxytropis sericea*. Oecologia **120:** 198-208

Yeh KW, Chen JC, Lin MI, Chen YM, Lin CY (1997) Functional activity of sporamin from sweet potato (*Ipomoea batatas* Lam): A tuber storage protein with trypsin inhibitory activity. Plant Molecular Biology **33:** 565-570

Yunes ANA, de Andrade MT, Sales MP, Morais RA, Fernandes KVS, Gomes VM, Xavier-Filho J (1998) Legume seed vicilins (7S storage proteins) interfere with the development of the cowpea weevil (*Callosobruchus maculatus* (F)). Journal of the Science of Food and Agriculture **76:** 111-116

Zangerl AR, Hamilton JG, Miller TJ, Crofts AR, Oxborough K, Berenbaum MR, de Lucia EH (2002) Impact of folivory on photosynthesis is greater than the sum of its holes. Proceedings of the National Academy of Sciences of the United States of America **99:** 1088-1091

Zavala JA, Baldwin IT (2006) Jasmonic acid signalling and herbivore resistance traits constrain regrowth after herbivore attack in *Nicotiana attenuata*. Plant Cell and Environment **29:** 1751-1760

Zavala JA, Patankar AG, Baldwin IT (2004) Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses. Plant Physiology **134:** 1181-1190

Zavala JA, Patankar AG, Gase K, Baldwin IT (2004) Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata*. Proceedings of the National Academy of Sciences of the United States of America **101:** 1607-1612

Zhang L, Cohn NS, Mitchell JP (1996) Induction of a pea cell-wall invertase gene by wounding and its localized expression in phloem. Plant Physiology **112:** 1111-1117

Zhou L, Jang JC, Jones TL, Sheen J (1998) Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. Proceedings of the National Academy of Sciences of the United States of America **95:** 10294-10299

Zou JP, Cates RG (1994) Role of douglas fir (*Pseudotsuga menziesii*) carbohydrates in resistance to budworm (*Choristoneura occidentalis*). Journal of Chemical Ecology **20:** 395-405

4. Discussion

The aim of this thesis was to study how primary metabolism is involved in plant resistance to herbivory, and to qualitatively evaluate the employment of reverse genetics in ecological research. This general discussion will cover the molecular regulation of plant tolerance of herbivory (**4.1**), the role of primary metabolism in plant resistance (defense and tolerance) to herbivory (**4.2**), and the use of reverse genetics in ecology (**4.3**).

4.1 Molecular regulation of plant tolerance of herbivory

Plant tolerance of herbivory has been studied for several decades, and several mechanisms have been suggested that may facilitate tolerance. Enhanced photosynthetic activity, activation of dormant meristems, altered architecture and resource allocation, or use of stored resources have been the most prominent explanations (Stowe et al., 2000). However, since it turned out to be very challenging to empirically verify mechanisms responsible for tolerance, as a consequence, most of the publications related to plant tolerance of herbivory present operational definitions rather than experimental evidence for either of these theoretical models (Tiffin, 2000).

In this thesis, it could be shown that in response to herbivory *N. attenuata* gains a measure of tolerance by allocating carbon to the roots, which is re-used for reproduction at the end of development, when herbivores are no longer a threat for the plant. This response is regulated by a subunit of a SnRK kinase, GAL83, and may be a generally beneficial strategy for annual plants that commonly do not accumulate large nutrient reserves in below-ground tissues during their development.

Interestingly, it was observed that after leaf wounding that was not associated with insect-specific elicitors, *N. attenuata* did not alter its carbon allocation but responded to the damage by increased branching. As a result, the seed capsule production was the same as in undamaged plants. Obviously, *N. attenuata* can initiate different compensatory strategies according to different types of damage. The response that is normally activated after leaves are wounded, increased branching, is “overwritten” by insect-specific elicitors, e.g. fatty acid–amino acid conjugates from oral secretions of *M. sexta*, leading to increased carbon allocation

to roots. This demonstrates that a plant's phenotype in response to different types of damage is highly plastic and that a plant can respond very specifically to a specific attacker.

A single gene, GAL83, the β-subunit of a SnRK kinase, was observed to have a significant effect on tolerance. SnRKs are known to be key regulators of primary metabolism in eukaryotes, particularly of carbon metabolism. The homologues of SnRK kinases in yeast, SNF1 kinases, and in mammals, AMPK kinases, are well studied. Their function in plants as central regulators of primary metabolism has only become obvious in recent years, and how they regulate metabolism and at which metabolic nodes they are active are not fully understood (Halford et al., 2004; Hardie, 2004). It is known that the activities of several enzymes of primary metabolism, such as sucrose synthase, nitrate reductase, HMGR carboxylase, and α-amylase are regulated by the complex. However, the details of the regulation of carbon allocation between root and shoot by GAL83 and the SnRK kinase, as seen in this study, remain to be unravelled. It is known that increased sink strength of roots is necessary for an increased carbon demand. This is carried out by invertases in sink tissues (Roitsch and Gonzalez, 2004) that cleave sucrose into glucose and fructose, thereby altering the osmotic gradient of sucrose between sources (source leaves: sucrose exporter) and sinks (roots: sucrose importer). In this study, we showed that the activity of vacuolar invertase is increased in roots in response to leaf wounding; however, only after application of insect-specific elicitors was carbon allocation to roots increased. It is possible that SnRK kinases activate sucrose transporters that regulate the export of sucrose from source leaves to roots.

An unresolved question is how plants activate increased root resources for reproduction. For example, undamaged plants silenced for GAL83 have a significantly greater root mass at the end of their development than do plants which were wounded or experienced herbivore damage at an early stage of their development; moreover, the seed capsule production of untreated asGAL83 plants is significantly lower than that of untreated wild types. Why do untreated asGAL83 plants not use their root reserves? Wounding or herbivore damage may induce a signal that allows the plant to "remember" that early and timely limited damage (in this case for 6 days) has to be compensated for at the stage of reproduction (ca. 60 days after treatment). Only after the different treatments are the root reserves in GAL83-silenced plants mobilized. How a plant coordinates this complex response remains unknown.

Several studies have addressed the question, do tolerance and defenses mutually exclude each other? I.e. does there exist a trade-off between those two traits, or can both traits occur simultaneously in a species (Tiffin, 2000)? Some authors suppose that natural selection will favor either one or the other: A fully tolerant plant will survive herbivore attack without

fitness losses, making defenses omittable; alternatively, a well-defended plant will have no need for compensation, because damage will be strongly reduced. Tolerance and defense, therefore, would exclude each other, and some examples suggest that defense is negatively associated with tolerance (Strauss and Agrawal, 1999). A trade-off could occur for several reasons. For example, costs of the two traits could restrict the simultaneous expression of both traits, if the combined costs add up to levels that would exceed a plant's energy and resource pools. However, even though defenses have been shown to be costly for a plant in terms of energy and resources, there is no evidence so far for such costs that are related to tolerance, making this argument unlikely. Furthermore, both traits could be genetically linked in a way that activating defenses would suppress tolerance-related genes and vice versa. This will be very difficult to prove, especially since parts of defense signalling and the genetic basis of tolerance are only poorly understood. Some researchers assume that, depending on the actual selection forces, a plant might favor either a more tolerant or a more defense-oriented strategy (Fineblum and Rausher, 1995; Van Der Meijden et al., 2000).

A mixture of both strategies could be reasonable for plants that do not suffer from only one herbivore or one type of damage during their development, since the simultaneous production of several different defenses, which are effective against different organisms, would increase costs dramatically. It is also likely that a plant's ability to defend a specific herbivore is restricted and therefore, it developed tolerance as a supportive trait. Thus, if selective pressure is affecting both types of response, it should be expected that different plants follow different strategies of excluding or mixing tolerance and defense, depending on their environment; under a specific selective pressure, a specific balance between defense and tolerance will be established. Many plant species are attacked during one growing season by several different herbivores from different feeding guilds, such as phloem feeders, mesophyll feeders, leaf eaters, root and stem feeders, or by necrotic and non-necrotic pathogens. A single resistance strategy would be insufficient to cope with all these different kinds of herbivores and pathogens, which cause substantially different types of damage. From this point of view, a combination of different strategies would be likely.

In a recent meta-analysis, Leimu and Koricheva (2006) reviewed trade-offs between tolerance and defense in a variety of plant species and found no negative correlation between them. That suggests that plants can follow both strategies in combination and that therefore plants are likely to respond highly plastic, resulting in variable combinations of defenses and tolerance, dependent on the type of damage. Thus, a joint evolution of defense and tolerance seems likely (Stowe et al., 2000).

Studies on coevolution of herbivores and plants have revealed that the development of enhanced plant defenses imposes selective pressure on herbivores; these then, as a population, respond by favoring mutations that allow them to cope with the new defenses. As a consequence, the plant has to improve its defenses again. However, it has not been shown yet that tolerance traits act as selective agent for herbivores to develop counter-active traits. Thus, tolerance would not be supportive in an evolutionary arms race that forces the herbivore to increase its damage potential. Tolerance, the 'Mahatma Gandhi-approach', because it breaks the spiral of an arms race by a defensive behavior instead of defenses, would therefore be an evolutionary advantage of the plant.

4.2 The role of primary metabolism in plant resistance to herbivory

The response of plants to herbivory has long been thought to result from the activation of specific "secondary" branches of metabolism that allow a plant to establish specific resistance traits. For example, the production of certain toxic compounds, such as alkaloids or glucosinolates, is induced after herbivore feeding, and a clear functional correlation has been empirically found between the compounds and their detrimental effects on herbivore performance. This is also found for volatile compounds that are released to attract predators of the herbivore, for proteinase inhibitors that disturb the insect's digestion, and for defense-related plant hormones. However, recent studies have investigated several new aspects of plant resistance that require a new view of how plants re-organize their metabolism in response to herbivory. It is becoming apparent that many genes and metabolites that are normally associated with primary metabolism, i.e. the part of the metabolism that is responsible for growth and reproduction of an unstressed plant, have functions in plant resistance. Obviously, the annotation of a gene or a metabolite to any part of the metabolism is not exclusive, and several genes and metabolites can have different functions at different levels of the organism's hierarchy.

Most of those metabolites were found because high levels of them accumulate in some plants but not in other plants, and hence are not assumed to be essential for growth and reproduction. Some genes were found because they showed the same herbivore-induced transcriptional activation levels that are seen in most secondary genes specifically related to resistance. For example, the part of primary metabolism that is related to storage of nutrients plays an important role in plant resistance. For example, vegetative storage proteins earned their name because they were thought to store amino acids and nitrogen due to their high

accumulation in various species. When those proteins were studied further, many of them were observed to exhibit enzymatic functions that could act defensively against insects (Berger et al., 2002; Liu et al., 2005). Another example is calcium oxalate (CaOx), which is found in high concentrations in several plant species. Its primary function is to provide Ca for cell metabolism and to maintain Ca homeostasis (Franceschi and Nakata, 2005). In some plants, CaOx forms large crystals that can physically protect certain tissues from chewing insects (Ruiz et al., 2002). Other plants control the crystallization of this salt in such a way that the crystals form barbed spikes.

Reorganizing how carbon and nitrogen are allocated is an important prerequisite for plant tolerance of herbivory. Normally, carbon and nitrogen are distributed within the plant according to the needs of different tissues for growth and reproduction. Once they are attacked by herbivores, plants reorganize this distribution in order to store nutrients in safe tissues that allow them to re-use those reserves after the herbivore pressure has ended. Leaf herbivory increases carbon allocation to roots, and root herbivory increases nitrogen allocation to shoots. This herbivore-induced reorganization may be regulated by the same "primary" genes that control nutrient allocation for growth and reproduction.

Several components of primary carbon metabolism are involved in signalling cascades that are elicited by herbivore feeding. Different sugars, sucrose, glucose, fructose and trehalose, and hexokinases have been shown to activate defensive genes or to regulate photosynthesis (Jang and Sheen, 1994, 1997; Halford and Paul, 2003; Paul, 2007). The orchestration of resistance responses depends on signalling compounds that are specifically activated by herbivores, such as WIPK, SIPK (Wu et al., 2007), and WRKYs (Eulgem and Somssich, 2007), as well as on signals that regulate metabolism of an unstressed plant. These complex interactions are only partly understood and can be studied by selectively silencing parts of the signalling pathways (Rook and Bevan, 2003); however, silencing genes that play primary roles causes some difficulties.

When genes that have primary functions are silenced to determine their functions in resistance, the resulting phenotype is likely related to alterating primary metabolism rather than to silencing the secondary functions of that gene. Therefore, an important criterion is that plant growth and reproduction must not be altered by silencing a specific gene, when the gene's resistance functions are to be found. It would be advantageous to silence such genes only locally in specific tissues or at specific time points; however, the actual gene silencing techniques are not yet sophisticated enough.

It has been shown that in some cases silencing a primary gene does not alter growth and reproduction. Silencing TD in *N. attenuata* produced plants with different grades of mRNA reduction, resulting in some plants that were altered in growth and others that were not, the so-called mild phenotypes (Kang et al., 2006). This was likely caused by pleiotropic effects that depended on the position of the integration site of the T-DNA in the genome. The RNAi silencing apparatus is not fully activated by the introduced DNA in some cases, and the primary function of the targeted gene is not disturbed. TD's function in defense depends on an mRNA level that is 30 times greater than the level that is necessary for primary functions. Thus, silencing TD in mild phenotypes did not eliminate TD mRNA completely, but prohibited the accumulation of large amounts of TD mRNA. These plants helped understanding the functions of TD in defense. It would be generally reasonable to test plants that underwent gene silencing for primary genes and have no altered phenotype in terms of growth and reproduction (and therefore might be classified as unsuccessfully transformed) for specific phenotypes that are related to herbivore resistance. Exposing these plants to their natural environment, which includes herbivores, is a powerful strategy to unravel secondary functions of primary genes.

4.3 Reverse genetics in ecological research

Advances in gene silencing during the last two decades have provided plant scientists with tools that have opened fundamentally new ways to study the functions of genes *in vivo*. The mechanisms that underlie *Agrobacterium*-mediated gene silencing are only recently beginning to be understood. Great progress has recently been made in unravelling the process of RNA interference that is responsible for gene silencing (Matzke and Birchler, 2005; Waterhouse, 2006). The improvements of the transformation procedures nowadays make transforming many plant species relatively easy. Thus, it is very likely that reverse genetics will soon become a standard tool for ecologists. The *in vivo* study of ecological functions of genes and their contribution to Darwinian fitness is of major interest when plant-herbivore systems are investigated.

However, the use of reverse genetics to study gene function is still criticized by some scientists due to possible unintended side-effects of the transformation procedure that are thought to be able to alter the genetic integrity of the transformed organism. A main concern is that these side-effects may alter the plant phenotype, including fitness-relevant traits. As a consequence, measured phenotypes of transformed plants may be related to the side-effects

and not to the silencing of a specific gene. If reverse genetics is to become a standard tool for ecologists, this technique must be proven to be reliable for the study of ecological gene function, without inducing effects, which may be detrimental to gene silencing. Unintended side-effects of transformation have been analyzed in detail on a molecular level, as the vast number of publications demonstrates, but, surprisingly the impacts of those side-effects on plant phenotypes and Darwinian fitness have rarely been studied. Molecular side-effects effects may occur regularly but to different degrees (Forsbach et al., 2003; Filipecki and Malepszy, 2006) and include somaclonal variation, when tissue culture is used (Larkin and Scowcroft, 1981), chromosomal rearrangements (Nacry et al., 1998), deletions of nucleic DNA (Latham et al., 2006), or altered patterns of DNA methylation (Wassenegger et al., 1994; Vanyushin, 2006; Zhang et al., 2006). Genome sequencing of each transformed plant could serve as a control for some of the side-effects of transformation; however, it is impossible to apply due to the extraordinary costs that are required for a sequencing result that is 100% correct.

Empty vector controls (EVCs) are another tool to test a transformation procedure for unintended effects that is much less costly, but they are rarely included in studies that use reverse genetics, probably because to do so requires additional and highly laborious experimental work. In this thesis, 5 independently transformed EVC lines of *N. attenuata* were tested for unintended alterations of their phenotypes (ecologically relevant traits and Darwinian fitness) that may have been caused by tissue culture and *A. tumefaciens* infection. To amplify subtle differences in plant performance, the experiments were carried out with a paired design: each plant line was paired with a wild-type plant in one pot. None of the 5 EVC lines exhibited any statistically significant differences compared to isogenic wild types for a battery of critical ecological parameters: defense traits, hormone levels, the transcriptional profile of 1,400 resistance- and primary metabolism-related genes in response to simulated herbivory, and Darwinian fitness, the crucial parameter in functional ecological studies, which was estimated by the number of seed capsules produced. Darwinian fitness reflects the total of all effects, positive and negative, on a plant's reproductive capacity during growth.

Any unmeasured molecular side-effects that might have occurred during tissue culturing and *Agrobacterium* transformation of EVC lines did not significantly influence any of these ecologically important traits. That no differences were observed between EVC lines and wild types may be related to the size of the genome of *N. attenuata*. For example, the genome of *N. tabacum* is 20 times larger than that of *Arabidopsis*, but the number of genes is

only twice as large. This strongly decreases the chance that T-DNA will be integrated into a coding region of the DNA compared to *Arabidopsis*, where coding sequences make up 44 % of the genome (Krysan et al., 2002).

The improvement of gene-silencing techniques will be advantageous for functional gene studies. Several attempts have been made to minimize the variation of transgene expression (Butaye et al., 2005). Floral dip, where flowers are dipped into a solution containing *Agrobacterium,* is a relatively simple and effective technique of transformation that is not dependent on tissue culture and callus induction (Labra et al., 2004). More effective gene silencing can be achieved by hormonally influencing the cell cycle (Arias et al., 2006). Of great interest is inducible RNA silencing (Lo et al., 2005), which has recently been described, as well as silencing gene families or parts of gene families (Miki et al., 2005; Zhao et al., 2005; Kaur et al., 2006). Newer constructs of transformation vectors allow the detection of the insertion site of the T-DNA in the genome. The flanking sequences can be determined and it can be tested, whether coding sequences of genes may be interrupted (Steppuhn et al., 2004).

5. Summary

Plant resistance against herbivory is dependent on a variety of transcriptional and metabolic responses that are elicited by tissue damage and insect-specific elicitors. Commonly, metabolism is separated in "primary" and "secondary", where "primary" is thought to be responsible for growth and reproduction of an unstressed plant, and "secondary" for specific responses to certain environmental stresses. Recent studies, however, suggest that a substantial part of a plant's primary metabolism is involved in herbivore resistance. Reverse genetics is a versatile tool that allows the functional study of genes *in vivo* and was used in this thesis to study the function of primary metabolism in plant tolerance against herbivory. All experiments have been conducted with the ecological model system *Nicotiana attenuata* (Solanaceae) and its specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae).

Tolerance of *N. attenuata* against damage of *M. sexta* is a result of altered carbon allocation that is controlled by a SNF1-related kinase (I). In response to herbivory, the β-subunit of a SNF1-related kinase, GAL83, is rapidly down-regulated in source (carbon exporting) leaves, and an increased portion of photoassimilates is transported to the roots. These newly accumulated resources can later be re-utilized when the herbivore pressure has ended and been translated into seed capsule production. It could be shown that this response is elicited by fatty acid-amino acid-conjugates that are found in oral secretions of *M. sexta.* A plant line that had reduced levels of the defense-related phytohormone jasmonic acid showed the same response as wild types, demonstrating that the tolerance mechanism is independent of jasmonic acid that regulates a majority of herbivore-induced changes in plants.

In this thesis the actual standard of knowledge about the roles of primary metabolism in plant resistance against herbivory was reviewed (III). Several aspects of resistance are related to primary metabolism: In order to fuel pathways that produce costly defensive compounds after herbivore attack, plants reorganize resource allocation patterns of carbon and nitrogen that are normally used for growth and reproduction. By changes of nutrient allocation or photosynthesis, activation of dormant meristems, or increased branching plants can tolerate tissue damage. Primary metabolites of sugar metabolism are involved in signaling pathways that are activated by herbivores and coordinate a plant's defense and tolerance responses. Moreover, several primary metabolites can have defensive functions. The recent findings that show the strong involvement of primary metabolism in resistance against herbivory demonstrate that the simple reductionistic view on how plants respond to their

environment has to integrate the complex and whole-organismic reorganisations of primary metabolism, multiple functions of single compounds and genes, as well as synergisms between secondary and/or primary metabolites.

Reverse genetics is a widely used technique for the study of genes *in vivo*, however, unintended side-effects on the genome may be caused by the common transformation procedure that is based on A*grobacterium* infection. In this thesis, it was tested, whether *Agrobacterium*-transformed plants that contain every information necessary for gene silencing except the silencing information itself (empty vector controls, EVCs), are different from wild types for a variety of ecologically relevant plant traits, Darwinian fitness (measured as seed capsule production), and transcription of 1,400 genes related to resistance and primary metabolism (II). It could be shown that EVC lines did not statistically differ from wild types for any measured trait. A power analysis revealed that several thousands of replicates would be necessary to detect statistically significant differences of fitness between EVC lines and wild types, but that 10 replicates were enough to detect statistically significant differences in fitness between wild types and plants that were silenced for a defense-gene, a trypsin proteinase inhibitor. These results demonstrate that transformation of *N. attenuata* with *Agrobacterium* is very unlikely to alter the phenotype in an unwanted manner, and that ecological studies using reverse genetics are not limited by possible side-effects of the transformation technique.

6. Zusammenfassung

Pflanzen haben im Laufe einer langen Koevolution mit Herbivoren verschiedene Mechanismen entwickelt, um das Ausmaß und die Auswirkungen von Biomassenschädigung und -verlust durch Herbivorie zu verringern. Direkte und indirekte Verteidigung reduzieren den Schaden an der Pflanze, indem Herbivoren mit mechanischer Abwehr (z. B. Dornen) abgewehrt, mit chemisch-physiologischer Abwehr (Nervengiften wie Nikotin oder verdauungshemmenden Proteinen, Proteaseinhibitoren) in ihrer Entwicklung gestört werden, oder indem die Pflanze durch Abgabe flüchtiger Verbindungen Fraßfeinde oder Parasitoiden von Herbivoren anlockt. Die Untersuchung dieser Mechanismen erfährt heute ein zunehmend starkes Interesse, weniger gut untersucht ist jedoch, inwieweit es Pflanzen möglich ist, nicht nur die Schädigung an sich, sondern auch die negativen Auswirkungen eines bereits eingetretenen Schadensereignisses abzumildern, oder sogar ein bestimmtes Ausmaß an Schaden ohne (Darwin´sche) Fitnessverluste hinzunehmen. Diese - im Allgemeinen als Toleranz bezeichnete - Pflanzenantwort kann auf unterschiedlichen Mechanismen beruhen, die größtenteils nur theoretisch beschrieben sind. Als zugrundeliegende Mechanismen werden verschiedene Anpassungen des Primärstoffwechsels diskutiert, wie z. B. die zusätzliche Aktivierung von „ruhenden“ Meristemen für ein kompensierendes Wachstum, eine erhöhte Photosyntheseaktivität, oder das Nutzen von schon vorhandenen oder kurzfristig angelegten Nährstoffspeichern aus verschiedenen Geweben.

Die Bedeutung von quantitative trait loci (QTLs), die Beteiligung von vielen Genen mit kleinen Effekten, wird von vielen Wissenschaftlern für Toleranz sehr hoch eingeschätzt, insbesondere, weil es bisher noch nicht möglich war, einem einzelnen Gen eine Funktion für Toleranz zuzuordnen. Reverse Genetics, das Erzeugen eines Phänotypen durch Gene Silencing, erlaubt die Analyse der Funktion eines Genes *in vivo*, und wurde in dieser Arbeit eingesetzt, um die Rolle von SnRK Kinasen in Bezug auf Toleranz von Herbivorie bei dem Modellsytem *Nicotiana attenuata* (Solanaceae) und dem darauf spezialisierten Herbivoren *Manduca sexta* (Lepidoptera, Sphingidae) zu untersuchen. Zusätzlich wurde die Anwendbarkeit der Technik des gene silencings evaluiert, um zu testen, ob mögliche molekulare Nebeneffekte des Transformierens ökologische Untersuchungen eines Genes stören können, indem phänotypische Merkmale unabsichtlich verändert werden.

Im Rahmen dieser Arbeit wurde ebenfalls ein Review angefertigt, der den aktuellen Stand der Forschung zur Bedeutung des Primärstoffwechsels für Verteidigung und Toleranz gegenüber Herbivorie beschreibt.

Toleranz gegenüber Herbivorie kann der Funktion eines Genes zugeordnet werden (Schwachtje et al., PNAS, 2006)

Pflanzen können Schädigung durch Herbivoren tolerieren. Im besten Fall löst Herbivorenbefall eine Überkompensation aus, die in einer erhöhten Darwin´schen Fitness gegenüber unbefallenen Pflanzen resultiert. Verschiedene Mechanismen für Toleranz werden diskutiert, es konnte bisher jedoch keine molekulare Regulation dafür gefunden werden. Anhand des ökologischen Modellsystems *Nicotiana attenuata* (Solanaceae) und dem Spezialisten *Manduca sexta* (Lepidoptera, Sphingidae), wurden die Mechanismen, die zu Toleranz gegenüber Herbivorie führen, auf molekularer Ebene untersucht. Folgende Ergebnisse konnten ermittelt werden:

- *N. attenuata* transportiert innerhalb weniger Stunden nach simuliertem Herbivorenbefall durch *M. sexta* (Blattverwundung mit Applikation von Raupenspeichel von *M. sexta*) vermehrt Kohlenstoff (Photoassimilate) in die Wurzeln (~ 10%). Dies konnte mit radioaktiv markiertem $^{11}CO2$ gezeigt werden.
- Die Kohlenstoffallokation geht einher mit reduzierten Mengen von löslichen Zuckern (Saccharose, Glukose, Fruktose) in Source-Blättern, sowie erhöter Aktivität von löslicher Invertase in den Wurzeln und ist unabhängig vom Hormon Jasmonsäure, das für die Regulation eines großen Teils der Resistenz gegen Herbivoren verantwortlich ist.
- Die Kohlenstoffallokation in die Wurzel ist reguliert durch die β-Untereinheit einer SnRK Kinase, GAL83, deren Transkripte innerhalb einer Stunde nach Herbivorenbefall in Source-Blättern deutlich vermindert sind. SnRK Kinasen sind zentrale Regulatoren des Zuckerstoffwechsels in Eukaryoten. Eine transgene Pflanze, in der GAL83 vermittels reverse genetics ausgeschaltet wurde, zeigte eine um 10% erhöhte Kohlenstoffakkumulation zur Wurzel, ohne dass Herbivorenbefall simuliert wurde.
- Die erhöhten Kohlenstoffmengen in den Wurzeln können von den Pflanzen gegen Ende ihrer Vegetationsperiode mobilisiert werden, und dienen einer vermehrten Produktion von Blüten und Samenkapseln. Mit Herbivorenbefall induzierte

Wildtypen, und nicht-induzierte transgene Pflanzen zeigten im Vergleich zum nicht-induzierten Wildtyp eine vermehrte Samenkapselproduktion gegen Ende ihrer Vegetationsperiode.

- Damit konnte erstmalig ein molekularer Faktor ermittelt werden, der direkt einen quantitativen Beitrag zur Toleranz von Herbivorie leistet, indem kurzfristig Ressourcen gespeichert werden, die später zur Samenproduktion zur Verfügung stehen.

Anwendbarkeit von Reverse Genetics in ökologischer Forschung (Schwachtje et al., accepted by PLoS ONE, 2007)

Seit ca. 20 Jahren ist es möglich, transgene Pflanzen herzustellen, in denen gezielt einzelne Gene ausgeschaltet sind. Erst in den letzten Jahren wurde der zugrundeliegende Mechanismus der RNA-Interferenz aufgeklärt. Meist wird die Information zur Gendeaktivierung (ein antisense oder inverted-repeat Fragment) mit *Agrobacterium tumefaciens* in die Pflanze eingeschleust. Während dieser künstlichen Infektion können verschiedene molekulare Effekte auftreten, die die ursprüngliche Struktur der DNA auf unerwünschte Weise verändern, was sich auf den Phänotyp der Pflanze auswirken kann. Kritiker wenden deshalb ein, dass für Experimente mit transgenen Pflanzen sowohl erhebliche Replikatanzahlen eingesetzt werden sollten, als auch mehrere unabhängig transformierte Linien, die mit „leeren“ Agrobacteriumplasmiden transformiert wurden (empty vector controls, evc). Diese enthalten lediglich die für die Transformation nötige genetische Information enthalten, nicht aber diejenige für Gene Silencing, was es erlaubt, mögliche Nebeneffekte des Transformierens zu erkennen.

In dieser Studie wurde untersucht, ob die Transformation von *Nicotiana attenuata* unerwünschte Effekte hervorruft, die groß genug sind, um ökologische Studien mit dieser Species stören können. Dazu wurden 5 unabhängig transformierte evc Pflanzenlinien (evc 1-5) sowohl mit Wildtypen verglichen, als auch mit einer „echten“ transformierten Linie (irPI), in der ein Gen für Proteinaseinhibitoren ausgeschaltet wurde. Die Ergebnisse dieser Studie sind wie folgt:

- Es konnten keine Unterschiede zwischen Wildtypen, evc 1-5 und irPI festgestellt werden in Bezug auf Herbivoren-induzierten Nikotingehalt und Produktion von Jasmonsäure, bzw. Jasmonsäure-Isoleucin, ein wichtiges Hormon für Signalübermittlung zur Aktivierung von Genen für Verteidigung. Unterschiedlich war wie zu erwarten die Aktivität von Proteinaseinhibitoren zwischen irPI und Wildtyp, bzw. evc 1-5, aber nicht zwischen evc 1-5 und Wildtyp.
- Mit Microarrays, die ca. 1500 Herbivorie- und Primärstoffwechsel-assoziierte Gene enthalten, konnte gezeigt werden, dass nach 24-stündiger Induktion der Pflanzen mit Methyl-Jasmonat, der Methylester des Verteidigungshormons Jasmonsäure, im Vergleich zum Wildtyp lediglich in der irPI-Linie 16 Gene signifikant unterschiedlich reguliert waren (davon 9 Proteinaseinhibtorsequenzen), nicht aber in den 5 evc Linien.
- Um Unterschiede bei der reproduktiven Fitness der Pflanzenlininen unter Nährstoffkonkurrenz festzustellen, wurden Pflanzen paarweise getopft (irPI, evc 1-5 und Wildtyp jeweils mit Wildtyp). Nach simuliertem Herbivorenbefall (Blattverwundung mit Applikation von Raupenspeichel von *M. sexta*) konnte in einem Experiment mit 20 Replikaten eine signifikant erhöhte Samenkapselproduktion bei der irPI Linie im Vergleich zu ihrem Wildtyp-Topfpartner festgestellt werden, nicht aber zwischen allen anderen Paaren.
- Die statistische Auswertung der Fitnessdaten ergab, dass 10 bis 15 Replikate ausreichen, um signifikante Unterschiede zwischen irPI und Wildtyp zu messen, dass jedoch, abhängig von der statistischen Methode (gepaarter t-Test für zwei Topfpartner oder ungepaarter t-Test für Unterschiede zwischen Paar-Differenzen) zwei bis drei Größenordnungen mehr Replikate notwendig sind, um Unterschiede zwischen evc und Wildtyp zu messen.
- Diese Ergebnisse zeigen, dass die Transformation von *N. attenuata* keinerlei messbare Auswirkung auf wichtige Verteidigungs-Parameter wie Nikotinproduktion, Proteinaseinhibitoraktivität, Produktion von Jasmonsäure bzw. Jasmonsäure-Isoleucin, die Regulation von knapp 1500 Genen, und die reproduktive Fitness (Anzahl der Samenkapseln) hat. Lediglich in der irPI Linie waren Unterschiede zum Wildtypen festzustellen. Diese sind alle assoziiert mit der Deaktivierung eines Proteinaseinhibitorgenes und waren beabsichtigt.

Funktionen des Primärmetabolismus bei der Herbivorenresistenz von Pflanzen (Review, Schwachtje und Baldwin, accepted by Plant Physiology, 2007)

Pflanzenstudien der letzten Jahrzehnte haben viele der grundlegenden Mechanismen aufgedeckt, die zu den verschiedenen Formen von Pflanzenresistenz gegen Herbivoren führen. Hierbei wurden die größten Fortschritte bei Studien von Metaboliten des Sekundärstoffwechsels gemacht, da diese relativ leicht zu untersuchen sind. Sekundärmetabolite können beispielweise Kunstfutter beigemischt werden, um Effekte auf Herbivoren zu untersuchen, oder es können gezielt Gene ausgeschaltet werden, die für die Synthese von Sekundärmetaboliten zuständig sind, um deren Effekt auf die Fitness der Pflanze und des Herbivoren *in vivo* zu messen.

Die Rolle des Primärstoffwechsels (hier definiert als der Teil des Stoffwechsels, der für Wachstum und Reproduktion einer ungestressten Pflanze verantwortlich ist) bei Herbivorenresistenz wurde bisher nur wenig untersucht. Gene oder Metaboliten, von denen eine Funktion im Primärstoffwechsel bekannt war, wurden nicht auf sekundäre Funktionen untersucht, weil es, dem - mittlerweile überholten - Paradigma „ein Gen - eine Funktion“ zufolge, ungewöhnlich erschien, dass ein Gen oder ein Metabolit mehrere Funktionen auf unterschiedlichen Ebenen der Organisationshierarchie einer Pflanze ausüben können. Seit allerdings neue Techniken zur Verfügung stehen, die eine umfassendere Analyse des Stoffwechsels und der Transkription nach Herbivorie ermöglichen, wie z. B. Microarrays, Metabolomics und Proteomics, gibt es vermehrt Hinweise darauf, dass ein relativ großer Anteil der nach Herbivorie regulierten Gene und Metaboliten dem Primärstoffwechsel zuzuordnen ist.

Es ist noch nicht möglich, Gene gezielt nur zu bestimmten Zeitpunkten oder nur in bestimmten Geweben auszuschalten. Bisher ist der Phänotyp einer Pflanze, in der ein Gen des Primärstoffwechsels ausgeschaltet ist, möglicherweise so stark verändert ist, dass die Untersuchung einer sekundären Genfunktion nicht mehr möglich ist und die gemessenen Fitnessparamter der Pflanze und des Herbivoren könnten aus der Veränderung des primären Stoffwechsels resultieren.

Ziel dieses Reviews war es, anhand von aktuellen Beispielen aus Signaltransduktion, Verteidigung und Toleranz aufzuzeigen, inwieweit Komponenten des primären Stoffwechsels an der Resistenz von Pflanzen gegen Herbivorie beteiligt sind, und mit welchen Methoden sekundäre Funktionen des Primärstoffwechsels aufgeklärt werden können.

6. References

Arias RS, Filichkin SA, Strauss SH (2006) Divide and conquer: development and cell cycle genes in plant transformation. Trends in Biotechnology **24:** 267-273

Baldwin IT (2001) An ecologically motivated analysis of plant-herbivore interactions in native tobacco. Plant Physiology **127:** 1449-1458

Bergelson J, Juenger T, Crawley MJ (1996) Regrowth following herbivory in *Ipomopsis aggregata*: Compensation but not overcompensation. American Naturalist **148:** 744-755

Berger S, Mitchell-Olds T, Stotz HU (2002) Local and differential control of vegetative storage protein expression in response to herbivore damage in *Arabidopsis thaliana*. Physiologia Plantarum **114:** 85-91

Broadway RM, Duffey SS (1986) Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exiqua*. Journal of Insect Physiology **32:** 827-833

Butaye KMJ, Cammue BPA, Delaure SL, De Bolle MFC (2005) Approaches to minimize variation of transgene expression in plants. Molecular Breeding **16:** 79-91

Cellini F, Chesson A, Colquhoun I, Constable A, Davies HV, Engel KH, Gatehouse AMR, Karenlampi S, Kok EJ, Leguay JJ, Lehesranta S, Noteborn H, Pedersen J, Smith M (2004) Unintended effects and their detection in genetically modified crops. Food and Chemical Toxicology **42:** 1089-1125

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666-U664

Cullen BR, Chapman DF, Quigley PE (2006) Comparative defoliation tolerance of temperate perennial grasses. Grass and Forage Science **61:** 405-412

Eulgem T, Somssich IE (2007) Networks of WRKY transcription factors in defense signaling. Current Opinion in Plant Biology **10:** 366-371

Filipecki M, Malepszy S (2006) Unintended consequences of plant transformation: a molecular insight. Journal of Applied Genetics **47:** 277-286

Fineblum WL, Rausher MD (1995) Tradeoff between resistance and tolerance to herbivore damage in a morning glory. Nature **377:** 517-520

Forsbach A, Schubert D, Lechtenberg B, Gils M, Schmidt R (2003) A comprehensive characterization of single-copy T-DNA insertions in the *Arabidopsis thaliana* genome. Plant Molecular Biology **52:** 161-176

Franceschi VR, Nakata PA (2005) Calcium oxalate in plants: Formation and function. Annual Review of Plant Biology **56:** 41-71

Gelvin SB (2003) *Agobacterium*-mediated plant transformation: The biology behind the "gene-Jockeying" tool. Microbiology and Molecular Biology Reviews **67:** 16-37

Halford NG, Hey S, Jhurreea D, Laurie S, McKibbin RS, Paul M, Zhang YH (2003) Metabolic signalling and carbon partitioning: role of Snf1-related (SnRK1) protein kinase. Journal of Experimental Botany **54:** 467-475

Halford NG, Hey S, Jhurreea D, Laurie S, McKibbin RS, Zhang YH, Paul MJ (2004) Highly conserved protein kinases involved in the regulation of carbon and amino acid metabolism. Journal of Experimental Botany **55:** 35-42

Halford NG, Paul MJ (2003) Carbon metabolite sensing and signalling. Plant Biotechnology Journal **1:** 381-398

Hardie DG (2004) The AMP-activated protein kinase pathway - new players upstream and downstream. Journal of Cell Science **117:** 5479-5487

Heil M, Koch T, Hilpert A, Fiala B, Boland W, Linsenmair KE (2001) Extrafloral nectar production of the ant-associated plant, *Macaranga tanarius*, is an induced, indirect, defensive response elicited by jasmonic acid. Proceedings of the National Academy of Sciences of the United States of America **98:** 1083-1088

Hrabak EM, Chan CWM, Gribskov M, Harper JF, Choi JH, Halford N, Kudla J, Luan S, Nimmo HG, Sussman MR, Thomas M, Walker-Simmons K, Zhu JK, Harmon AC (2003) The *Arabidopsis* CDPK-SnRK superfamily of protein kinases. Plant Physiology **132:** 666-680

Jang JC, Sheen J (1994) Sugar Sensing in Higher-Plants. Plant Cell **6:** 1665-1679

Jang JC, Sheen J (1997) Sugar sensing in higher plants. Trends in Plant Science **2:** 208-214

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology **43:** 179-188

Kang JH, Wang L, Giri A, Baldwin IT (2006) Silencing threonine deaminase and JAR4 in *Nicotiana attenuata* impairs jasmonic acid-isoleucine-mediated defenses against *Manduca sexta*. Plant Cell **18:** 3303-3320

Kaur J, Sebastian J, Siddiqi I (2006) The *Arabidopsis*-mei2-like genes play a role in meiosis and vegetative growth in *Arabidopsi*s. Plant Cell **18:** 545-559

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science **291:** 2141-2144

Kessler A, Baldwin IT (2002) *Manduca quinquemaculata*'s optimization of intra-plant oviposition to predation, food quality, and thermal constraint's. Ecology **83:** 2346-2354

Krock B, Schmidt S, Hertweck C, Baldwin IT (2002) Vegetation-derived abscisic acid and four terpenes enforce dormancy in seeds of the post-fire annual, *Nicotiana attenuata*. Seed Science Research **12:** 239-252

Krysan PJ, Young JC, Jester PJ, Monson S, Copenhaver G, Preuss D, Sussman MR (2002) Characterization of T-DNA Insertion Sites in *Arabidopsis thaliana* and the Implications for Saturation Mutagenesis. OMICS **6:** 163-174

Labandeira CC, Dilcher DL, Davis DR, Wagner DL (1994) Ninety-seven million years of angiosperm-insect association: Palaeobiological insights into the meaning of coevolution. Proceedings of the National Academy of Sciences of the United States of America **91:** 12278-12282

Labra M, Vannini C, Grassi F, Bracale M, Balsemin M, Basso B, Sala F (2004) Genomic stability in Arabidopsis thaliana transgenic plants obtained by floral dip. Theoretical and Applied Genetics **109:** 1512-1518

Larkin PJ, Scowcroft WR (1981) Somaclonal variation - a novel source of variability from cell-cultures for plant improvement. Theoretical and Applied Genetics **60:** 197-214

Latham JR, Wilson AK, Steinbrecher RA (2006) The mutational consequences of plant transformation. Journal of Biomedicine and Biotechnology**:** Art. No. 25376 22006

Leimu R, Koricheva J (2006) A meta-analysis of tradeoffs between plant tolerance and resistance to herbivores: combining the evidence from ecological and agricultural studies. Oikos **112:** 1-9

Liu YL, Ahn JE, Datta S, Salzman RA, Moon J, Huyghues-Despointes B, Pittendrigh B, Murdock LL, Koiwa H, Zhu-Salzman K (2005) *Arabidopsis* vegetative storage protein is an anti-insect acid phosphatase. Plant Physiology **139:** 1545-1556

Lo C, Wang N, Lam E (2005) Inducible double-stranded RNA expression activates reversible transcript turnover and stable translational suppression of a target gene in transgenic tobacco. Febs Letters **579:** 1498-1502

Mabry CM, Wayne PW (1997) Defoliation of the annual herb *Abutilon theophrasti*: Mechanisms underlying reproductive compensation. Oecologia **111:** 225-232

Matzke MA, Birchler JA (2005) RNAi-mediated pathways in the nucleus. Nature Reviews Genetics **6:** 24-35

Mewis I, Tokuhisa JG, Schultz JC, Appel HM, Ulrichs C, Gershenzon J (2006) Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. Phytochemistry **67:** 2450-2462

Miki D, Itoh R, Shimamoto K (2005) RNA silencing of single and multiple members in a gene family of rice. Plant Physiology **138:** 1903-1913

Nacry P, Camilleri C, Courtial B, Caboche M, Bouchez D (1998) Major chromosomal rearrangements induced by T-DNA transformation in *Arabidopsis*. Genetics **149:** 641-650

Paul M (2007) Trehalose 6-phosphate. Current Opinion in Plant Biology **10:** 303-309

Roda A, Nyrop J, English-Loeb G, Dicke M (2001) Leaf pubescence and two-spotted spider mite webbing influence phytoseiid behavior and population density. Oecologia **129:** 551-560

Roitsch T, Gonzalez MC (2004) Function and regulation of plant invertases: sweet sensations. Trends in Plant Science **9:** 606-613

Rook F, Bevan MW (2003) Genetic approaches to understanding sugar-response pathways. Journal of Experimental Botany **54:** 495-501

Ruiz N, Ward D, Saltz D (2002) Calcium oxalate crystals in leaves of Pancratium sickenbergeri: constitutive or induced defence? Functional Ecology **16:** 99-105

Ryan CA, Pearce G (2003) Systemins: A functionally defined family of peptide signal that regulate defensive genes in Solanaceae species. Proceedings of the National Academy of Sciences of the United States of America **100:** 14577-14580

Schmeltz I (1971) Nicotine and other tobacco alkaloids. *In* M Jacobson, DG Crosby, eds, Naturally Occurring Insecticides. Mercel Dekker, New York, pp 99-136

Schwachtje J, Baldwin IT (2004) Smoke exposure alters endogenous gibberellin and abscisic acid pools and gibberellin sensitivity while eliciting germination in the post-fire annual, *Nicotiana attenuata*. Seed Science Research **14:** 51-60

Staswick PE, Papa C, Huang JF, Rhee Y (1994) Purification of the major soybean leaf acid-phosphatase that is increased by seed-pod removal. Plant Physiology **104:** 49-57

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. PLOS-Biology **2:** 1074-1080

Stowe KA, Marquis RJ, Hochwender CG, Simms EL (2000) The evolutionary ecology of tolerance to consumer damage. Annual Review of Ecology and Systematics **31:** 565-595

Strauss SY, Agrawal AA (1999) The ecology and evolution of plant tolerance to herbivory. Trends in Ecology & Evolution **14:** 179-185

Tiffin P (2000) Mechanisms of tolerance to herbivore damage: what do we know? Evolutionary Ecology **14:** 523-536

Tzfira T, Citovsky V (2006) Agrobacterium-mediated genetic transformation of plants: biology and biotechnology. Current Opinion in Biotechnology **17:** 147-154

Van Der Meijden E, De Boer NJ, Van Der Veen-Van Wijk CAM (2000) Pattern of storage and regrowth in ragwort. Evolutionary Ecology **14:** 439-455

Vanyushin BF (2006) DNA methylation and epigenetics. Russian Journal of Genetics **42:** 985-997

Vincent O, Townley R, Kuchin S, Carlson M (2001) Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. Genes & Development **15:** 1104-1114

Wassenegger M, Heimes S, Riedel L, Sanger HL (1994) RNA-directed *de novo* methylation of genomic sequences in plants. Cell **76:** 567-576

Waterhouse PM (2006) Defense and counterdefense in the plant world. Nature Genetics **38:** 138-139

Wu JQ, Hettenhausen C, Meldau S, Baldwin IT (2007) Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*. Plant Cell **19:** 1096-1122

Zhang XY, Yazaki J, Sundaresan A, Cokus S, Chan SWL, Chen HM, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, Ecker JR (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in *Arabidopsis*. Cell **126:** 1189-1201

Zhao WZ, Fanning ML, Lane T (2005) Efficient RNAi-based gene family knockdown via set cover optimization. Artificial Intelligence in Medicine **35:** 61-73

7. Acknowledgements

I want to thank

- Ian T. Baldwin for taking me deep into science and for guiding me and my work in such a passionate and creative way during the last years. It was a great pleasure and an outstanding experience to work at his MPI department in Jena.
- Peter E. H. Minchin from HortResearch, New Zealand, Sigi Jahnke and Uli Schurr from the Phytosphäre in Jülich for giving me the chance to combine physics and biology in my experiments, which was an exciting interdisciplinary approach.
- Joost van Dongen from the MPI for Molecular Plant Physiology in Golm for sharing his knowledge about sugar metabolism.
- Wibke Kröber, Silke Allmann and Susan Kutschbach for helping me with a lot of microarrays and other things.
- Bernd Krock, Albrecht Berg, Matthias Schöttner, Eva Rothe, Michael Reichelt, and Stefan Bartram for helping with measurements of various metabolites on different sophisticated machines.
- Emily Wheeler for her kind editorial help with a lot of texts.
- the people from the greenhouse, Tamara Krügel, Andreas Schünzel, Andreas Weber, and all the gardeners for taking care of the several thousands of *N. attenuata* plants that I maltreated so badly, which I regret here.
- the members from all over the world of the Baldwin department that made the time there so enjoyable: Markus Hartl, Caroline von Dahl, Dominik Schmidt, Silvia Schmidt, Anja Paschold, Merijan Kant, Stefan Meldau, Holger Merker, Celia Diezel, Danny Kessler, and all the other actual and former collegues.
- my parents, grandparents and my brother for all kinds of help and for being interested in what I do.
- Anke for love, patience and help.
- Johanna and David for the future.

8. Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität zu Jena erkläre ich, daß ich die voriegende Promotion eigenständig angefertigt und alle von mir benutzten Hilfsmittel angegeben habe. Personen, die mich bei der Erhebung und Auswahl des Materials sowie bei der Erstellung der Manuskripte unterstützt haben, sind in der Auflistung der Manuskripte genannt. Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen noch haben Dritte für Arbeiten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Dienstleistungen erhalten. Die vorgelegte Dissertation wurde weder als Prüfungsarbeit für eine staatliche oder andere Prüfung, noch als Dissertation an einer anderen Hochschule eingereicht.

Jens Schwachtje

Jena, 1.12.07

10. Scientific publications and talks

Scientific publications

Schwachtje J; Minchin PEH; Jahnke S; van Dongen J; Schittko U; Baldwin IT (2006): SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. Proceedings of the National Academy of Sciences of the United States of America 103, 12935-12940

Schwachtje J; Baldwin IT (2004): Smoke exposure alters endogenous gibberellin and abscisic acid pools and gibberellin sensitivity while eliciting germination in the post-fire annual, Nicotiana attenuata. Seed Science Research 14, 51-60

Schwachtje J; Vorwieger A; Jain A; Singh A; Punia MS; Behl RK; Bergmann H (2002): Effect of sodium chloride on seed germination and other growth factors in soybean (Glycine Max L.). National Journal of Plant Improvement 4, 9-12

Talks (invited)

„The role of SnRK1 kinases for plant tolerance against herbivory"

Institut für Chemie und Dynamik der Geosphäre

ICG II: Phytosphäre Froschungszentrum Jülich, Jülich, Germany, May 2005

Talks

"Plant tolerance against herbivory"

IMPRS Evaluation Symposium / MPI for Chemical Ecology, Jena, Germany,

Sep 2007

"How plants can tolerate herbivory"

ICE Seminar, MPI for Chemical Ecology, Jena, Germany, 2006 Jun 01

"An SnRK kinase facilitates tolerance for herbivory by increasing C allocation to roots"
4th Biannual IMPRS Symposium / MPI for Chemical Ecology, Jena, Germany, Mar 2006

" Carbon Allocation and Plant Defense - The Crucial Role of a SnRK1 Kinase Complex"R
ICE Symposium / MPI for Chemical Ecology, Jena, Germany, Jul 2005

"GAL83, a subunit of a SnRK1 kinase complex, is involved in altered allocation of photoassimilates in herbivore-attacked *Nicotiana attenuata*"
2nd Biannual IMPRS Symposium / MPI for Chemical Ecology, Jena, Germany, Mar 2005

11. Appendices

Manuscript I Supplemental Material

Plant Transformation

N. attenuata wild types were transformed with *Agrobacterium* (strain LBA4404) as described in (47) by using a 391 bp antisense (*as*) construct to silence *N. attenuata* GAL83 (accession nr. AY460336). The *as* sequence was created with primers GAL1-32 5' GCGGCGGGTCACCGGCAGGTCAGGTCCACCCC and GAL2-34 5' GCGGCGCCATGGCACAGAGAATGAATATGCATTG with *N. attenuata* GAL83 as template. The PCR-fragment was digested with partial BstEII x NcoI and cloned into pNATGUS3, which was digested with BstEII x NcoI. 20 independently transformed lines were screened for homozygosity using Nourseothricin resistance as a selective marker. Single inserts were confirmed by Southern blots hybridized with a GAL83 PCR fragment and expression levels were determined by real-time RT PCR for 6 lines (see below), 2 of which were tested for root:shoot ratios (see below). All transformed plants were diploid as determined by flow cytometry (Bubner *et al.* 2006) . In another study we demonstrated that empty vector control lines of pNAT were not different from wild types in a variety of defense traits.

RNA Extraction and Determination

RNA extraction was carried out after the TRI reagent protocol (Sigma, Taufkirchen, Germany), using ground (pestle and mortar) and homogenized (Ultra-Turrax, T 25 basic, IKA, Staufen, Germany) tissue samples. Real-time RT-PCR was performed with the qPCR™ kit (Eurogentec, Seraing, Belgium) and analysis was carried out on an SDS7700 (Applied Biosystems, Darmstadt, Germany) as described by Halitschke *et al*. (2003). A specific probe for GAL83, which attached to a region different than that used for the antisense construct, was used to determine endogenous mRNA levels. Transcript accumulation was determined by using the following primers and fluorescence dye-labeled probe: GAL83for 5' CCCAGTAGTTCCTTTACAAGC, GAL83rev 5' CTCTGGAGAATGATCAGAGGC, probe: 5' TGGCCATCCTGCTACCGACCAAA. Endogenous expression levels of GAL83 in *as*GAL83 plant leaves reached 22 % of WT levels during the day before sinking to 4 % of WT levels during the night.

GAL83 RNA expression in *N. attenuata* is rapidly down-regulated (< 1 h) in source leaves by 60-70% in response to wound + regurgitant (R) treatment, detected by Northern blots and microarrays.

Plant Growth

Wild type (WT) plants were from a *Nicotiana attenuata* (synonymous with *Nicotiana torreyana*, Nelson and Macbr., Solanaceae) inbred line (17 generations) of a seed collection from a native population in Utah, USA. Plants for the ^{11}C experiments were grown in conical 1L pots, 18 cm tall, in a climate chamber under conditions as described in Krügel *et al*. (2002). Plants for enzyme activity and sugar measurements, RNA/DNA extraction, and root:shoot mass ratio measurements were grown in a hydroponic culture in a climate chamber as described in Krügel *et al*. (2002). All plants were grown in a climate chamber under the following conditions: 28°C/16 h light, 25°C/8 h dark, and 800 to 1,000 µmol m-2 s-1 PAR at plant height from high-pressure sodium lamps. Plants for Northern blots and the long-term glasshouse experiment were grown in 1 L pots in a glasshouse with supplemental lighting (Philips Sun-T Agro 400 or 600 W Na lights; Phillips Lighting, Somerset, NJ, USA) under the following conditions: 28 C/16 h (6 am – 10 pm) light, 22C/8 h (10 pm – 6 am) dark.

Plant Treatments

For Northern blotting (Fig. 4), plants were divided into four groups of 27 replicate plants. Plants from one group were treated on their second fully expanded (source) leaf at node +2. Plants from the other groups were treated either on the sink leaf at node -2 or on the orthostichous source leaf at node +6 (see leaf numbers in Fig. 1 A), or not treated at all (control group), every 30 min. With a fabric pattern wheel we created one row of puncture wounds parallel to the mid-rib every 30 min, and immediately added 5 µl of *M. sexta* regurgitant [collected from fifth-instar *Manduca sexta* larvae grown on wild type *N. attenuata* plants], diluted with water (1:3, v:v) to the wound sites. A total of eight rows -- four on each side of the middle rib -- were generated per leaf. Leaves at nodes -2, +2, and +6 were harvested from all four treatment groups; tip leaves (-3 and younger) were also collected as one bulk sample (27 plants) from each plant. All leaves were frozen in liquid nitrogen 30 min after the last treatment, except for the tip leaves, which were harvested 60 min after the last wounding event. Total cellular RNA was isolated from leaves bulk collected from 27 plants for each treatment group.

For ^{11}C-measurements (Figs. 1, 2), plants were elicited by puncturing source leaves with a fabric pattern wheel (2 lines on each side of the mid vein while keeping the amount of wounding identical among treatments) and immediately applying 20 µl deionized water, 20 µl water, regurgitant or water and FAC [fatty acid-amino acid conjugates, *N*-linolenoyl-L-Gln (50 ng mL^{-1}, 0.12 mM) and *N*-linolenoyl-L-Glu (138 ng mL^{-1}, 0.34 mM)], which are known to be the main elicitors of plant defense responses in *M. sexta* regurgitant.

For the long-term experiment (Figs. 5, 6), 15 plants per treatment and line (WT and *as*GAL83) were treated 6 days before elongation during rosette-stage growth (when all plants were at the same developmental stage) twice per day at 10 am and 4 pm by puncturing with a fabric pattern wheel and immediately applying deionized water or R. Each day, 4 different source leaves were treated (2 in each treatment), eliciting all orthostichious connections within the plant (Schittko *et al*. 2003). Furthermore, 10 rosette-stage plants per line were exposed to *M. sexta* feeding for 6 days: 4 neonate (freshly hatched) *M. sexta* larvae were placed on 4 different source leaves and allowed to feed freely for 3 days until they reached the second instar, after which they were replaced by 4 other neonates that again were removed after 3 days. We used this 2 x 3 day treatment to prevent larvae from moving within the plant, as they commonly do once they reach the second instar, and to minimize differences in the amount of damage between WT *as*GAL83 lines. The similarity of WT R and H treatments (Fig. 9) demonstrates that treating puncture wounds *M. sexta* regurgitant simulates the long-term growth effects of *M. sexta* feeding, as it has been shown for short-term transcriptional responses.

Plants were watered twice a day (with a nutrient-containing solution on ebb-and-flow glasshouse growing tables, which were flooded for 4 min during each cycle) and watering was stepwise reduced to zero over a ten-day period that started on day 33 and lasted until day 42 after end of the elicitation treatments, according to the following schedule:
day 33 – day 35: 50 %; day 36: no watering; day 37: 50 %; day 38: no watering; day 39: 50 %; day 40-41: no watering; day 42: 50 %.

^{11}C measurements

Experiments with the short-lived isotope 11C were carried out at the Phytosphere Laboratories of the Forschungszentrum Jülich (Germany) where ^{11}C is created by means of the nuclear reaction [^{14}N(p,α)^{11}C] within a cyclotron. The advantage of ^{11}C as a tracer is its half-life of 20.4 min and *in vivo* measurement, allowing partitioning of recently fixed

photosynthate to be measured non-invasively on the same plant several times per day. Within the labeled leaf, $^{11}CO_2$ is photosynthetically fixed to produce labeled sucrose, which is subsequently transported by the phloem to the sink tissues. ^{11}C labeling allows for real-time monitoring of these phloem fluxes using scintillation counters. When sink leaves (those growing at nodal positions -1 to -4 of early flowering stage plants, not yet fully expanded and still importing more assimilates than they export) were punctured and the puncture wounds treated with water (WS, Fig. 2 A), partitioning of ^{11}C-labeled assimilates to the roots increased significantly, whereas when source leaves were treated, there was no effect (Fig. 2 A). This was most likely due to the damage to phloem transport in sink leaves, which decreased sink strength. To study the signaling-induced physiological effects of our treatments rather than the physical effects of wounding, we treated source leaves, which are the preferred oviposition sites of adult *Manduca* in native *N. attenuata* populations. Plants at the rosette stage were placed into the experimental cabinet (25°C, 50 % humidity, 16 h/8 h day/night) a day before experiments. A leaf chamber was placed at the tip of source leaf +3, sealed and connected to ambient air (350 ppm CO_2, 1.5L/min flow rate) the day before tracer application, to allow the plant time to settle after handling. Precisely aligned lead (or tungsten) shielding was used to give accurate measurement of above-ground (entire shoot minus the labelled leaf blade) and below-ground (entire root) ^{11}C activity (see Fig. 1 B, C). $^{11}CO_2$ was introduced to leaf +3, with leaves +2, +4, and +5 being treated (see Fig. 1 A). Source leaves were treated twice, at 11 am and 2 pm, by puncturing the lamina with a fabric pattern wheel and immediately applying deionized water, *M. sexta* regurgitant, or synthetic FACs. $^{11}CO_2$ was applied at 9:30 am (i.e., before a leaf treatment) and at 2:30 pm (i.e., after leaf treatment). The shoot-to-root partitioning of ^{11}C-photosynthate was calculated from the observed tracer profiles (Minchin *et al*. 2002) and expressed as root and shoot percentage of total C activity, after which post and pre-treatment values were ratioed.

Cited Literature

Krugel T, Lim M, Gase K, Halitschke R, Baldwin IT (2002) *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. Chemoecology 12: 177.

Bubner B, Gase K, Bergner B, Baldwin IT Frequency of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue. Plant Cell Rep. In press.

Schittko U, Baldwin IT (2003) Constraints to herbivore-induced systemic responses: Bidirectional signaling along orthostichies in *Nicotiana attenuata*. J. Chem.Ecol. 29: 763-770.

Minchin PEH, Thorpe MR, Farrar JF, Koroleva OA (2002) Source-sink coupling in young barley plants and control of phloem loading. J Exp Bot 53, 1671-1676.

Figures

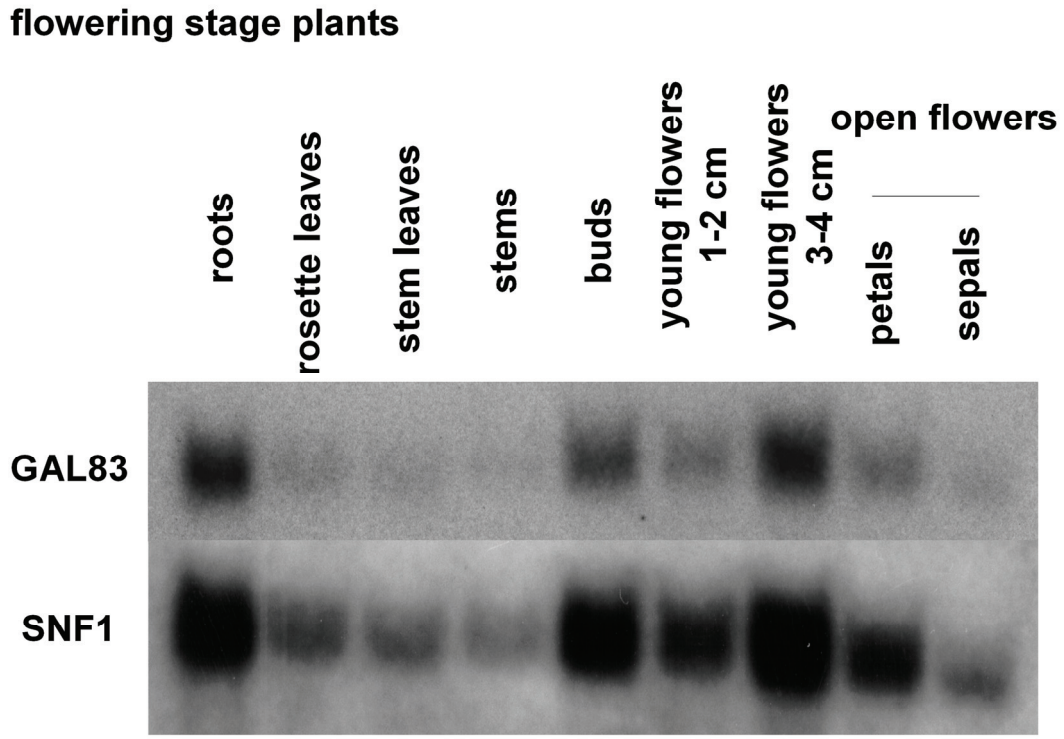


Figure 7. Expression of GAL83 and SNF1 in different tissues of a flowering *N. attenuata* plant, showing that both subunits are co-expressed in a similar ratio in all tissues where they occur.

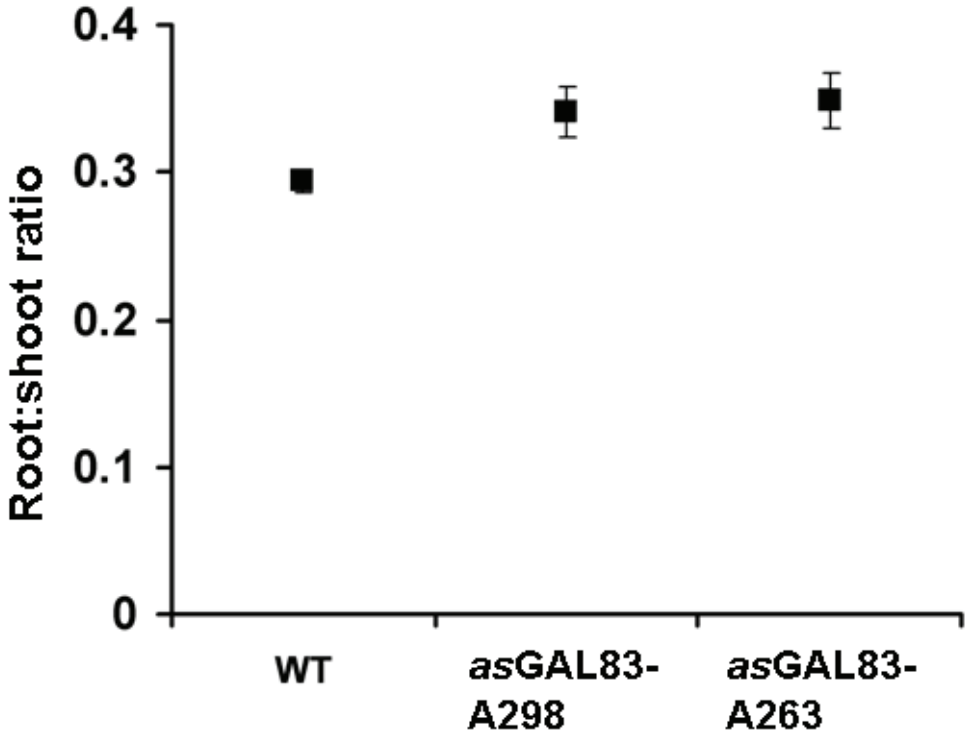


Figure 8. Root:shoot ratios (dry mass) of WT and two independently transformed *as*GAL83 lines. Hydroponically grown plants were harvested at early flowering stages. Transgenic lines had a greater root:shoot ratio than did WT (Mann Whitney, $P < 0.02$). All lines had the same total mass (Kruskal Wallis test, $H = 4.774$, $P > 0.05$).

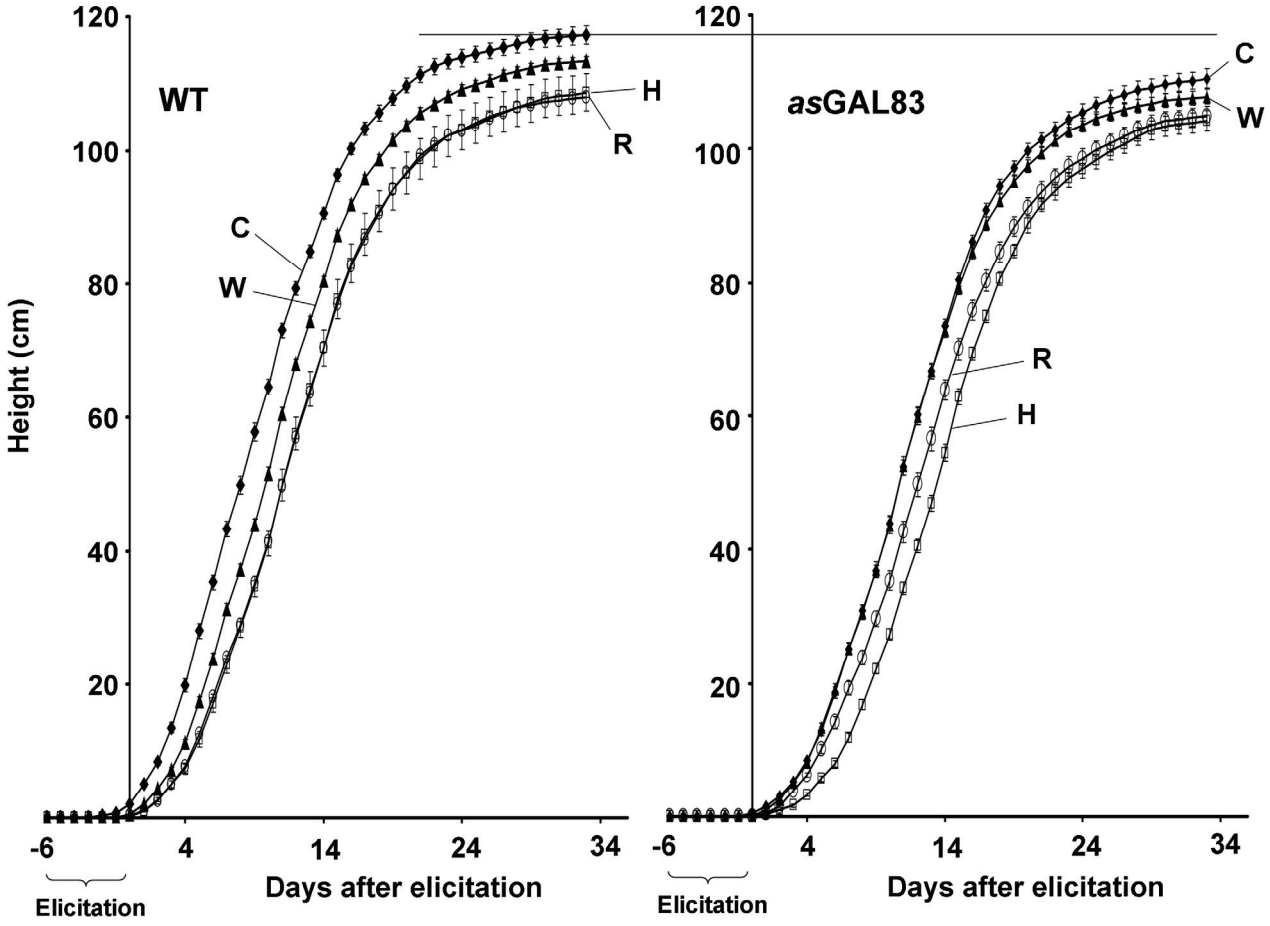


Figure 9. Height of plants [mean ± SE, n(C, W, R) = 15, n(H) = 10] during long-term glasshouse experiment. Left: WT, right: *as*GAL83.

t-tests of final heights:

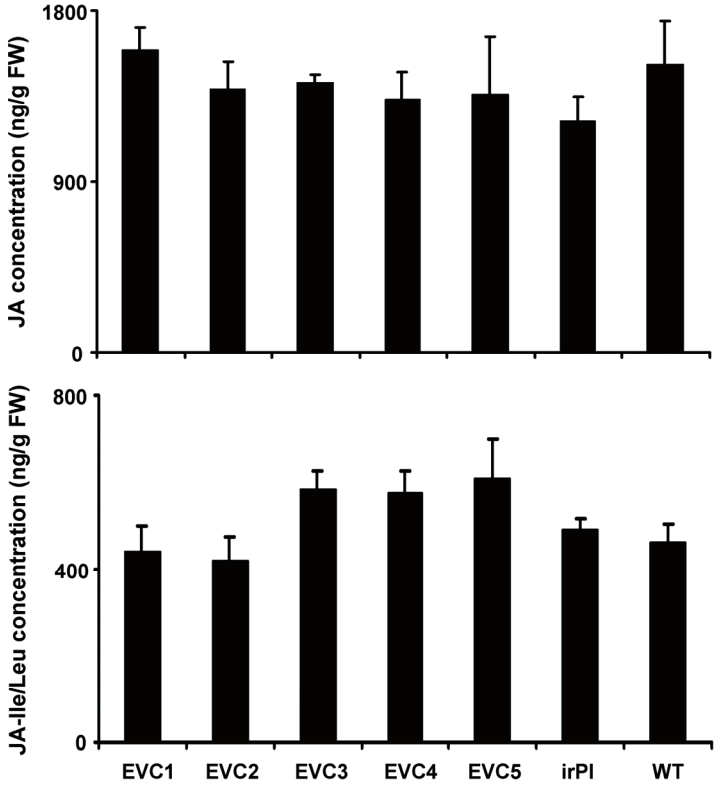
WT C vs. *as*GAL83 C: P = 0.0082, t = - 2.864

WT W vs. *as*GAL83 W: P = 0.0006, t = - 3.920

WT R vs. *as*GAL83 R: P = 0.2024, t = - 1.308

WT H vs. *as*GAL83 H: P = 0.2660, t = - 1.153

Manuscript II Supplemental Material



Supplemental Fig. 1: Levels of JA (upper graph) and JA-Ile/Leu conjugates (lower graph) in source leaves 45 min after elicitation with oral secretions of *M. sexta*. Means + SE. No statistically significant differences could be detected.